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14. ABSTRACT We have previously shown that an AR/EGFR autocrine loop is required for SUM149 human breast cancer cell proliferation, motility and invasion. In the present studies, we demonstrate that SUM149 cells and human mammary epithelial MCF10A cells that over express AR (MCF10A AR) or are cultured in the presence of exogenous AR, express higher levels of EGFR protein compared with MCF10A cells cultured in EGF. We show that EGFR protein remains stable in the presence of AR yet is degraded in the presence of EGF. Consistent with this observation, tyrosine 1045 on the EGFR, the c-cbl binding site, exhibited decreased phosphorylation in the presence of AR compared with EGF. Ubiquitination of EGFR was also dramatically decreased when AR was the ligand. Following AR binding, EGFR remained on the cell surface instead of being rapidly internalized as observed when EGF was present. Immunofluorescence demonstrated that MCF10A cells cultured in EGF exhibited a predominantly intracellular, punctate localization of EGFR. In stark contrast, SUM149 cells and MCF10A cells growing in the presence of AR expressed EGFR predominantly on the membrane and at cell-cell junctions. Therefore, AR alters EGFR internalization and degradation in a way that favors accumulation of EGFR at the cell surface and ultimately leads to changes in EGFR signaling. In addition, we found that AR, but not EGF upregulates NFkB activity and IL-1 production suggesting that AR may play a unique role in breast cancer progression.

15. SUBJECT TERMS
Amphiregulin, Epidermal Growth Factor Receptor, Autocrine Loop, Breast Cancer

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Nicole E. Willmarth
Annual Summary for Award W81XWH-06-1-0405
March 19, 2007

Introduction:

The epidermal growth factor receptor (EGFR) is a transmembrane receptor tyrosine kinase (1). The seven different ligands that can bind the EGFR are epidermal growth factor (EGF), transforming growth factor alpha (TGF-α), amphiregulin (AR), heparin binding EGF (HB-EGF), betacellulin (BTC), epiregulin (EPR), and epigen (2-8). Once ligand binds, the receptor dimerizes and becomes activated, leading to propagation of downstream signals (9). These downstream signals are involved in a number of normal cellular processes such as proliferation, differentiation, migration, and survival. Given the pleiotropic effects of EGFR signaling, the duration and strength of EGFR signals must be tightly controlled as dysregulated EGFR signaling has been demonstrated to contribute to tumorigenesis (10).

Modulation of receptor trafficking and degradation are essential for proper EGFR signaling. It has been shown in several cell types that EGFR is rapidly internalized and degraded upon activation by EGF (11-13). The ubiquitin ligase responsible for targeting EGFR for degradation is c-Cbl. c-Cbl is an E3 ubiquitin ligase that contains a phosphotyrosine binding domain at its N-terminus as well as a C3HC4 RING finger (14). c-Cbl can bind the EGFR either directly via the pY1045 site on the receptor via its tyrosine kinase binding domain or indirectly via binding to Grb2 (15-17). However, at high but physiological concentrations of EGF, the former interaction is predominant (16, 18-20). c-Cbl recruits ubiquitin conjugating enzymes to transfer ubiquitin on to the receptor via its RING finger domain. In this manner, c-Cbl positively regulates clathrin-dependent endocytosis of EGFR. Ubiquitination of EGFR by c-Cbl is required for directing the EGFR to late endosomes and then for lysosomal degradation (12). Over expression of c-Cbl significantly increases EGF-induced EGFR degradation (11). If EGFR is not targeted for ubiquitination and degradation by c-Cbl, it is recycled back to the surface via recycling endosomes where it can be activated again (16). Therefore, c-Cbl mediated ubiquitination plays a critical role in the regulation of EGFR signaling.

The majority of EGFR trafficking studies have been performed using EGF as the ligand (11, 12, 21). Therefore, much of what we know is based on how EGF interacts with and regulates receptor recycling and degradation. However, it has been demonstrated in some studies that the diversity of EGF ligands can lead to a unique interaction with the receptor which affects intracellular trafficking of the ligand/EGFR complex (13, 22). In one study EGF was found to bind tightly to the receptor, resulting in EGFR degradation. In contrast, TGF-α was found to bind less tightly and consequently it completely dissociated at the lower pH of the endosomal compartment which lead to receptor recycling. Single amino acid substitutions in EGF decreased its binding to the receptor and as a result, decreased the degradation rate of EGFR compared with EGFR activated by wild type EGF (13). Thus, different ligands can have diverse effects on the regulation of EGFR trafficking.

The effects of the EGF family ligand AR on EGFR trafficking or degradation have not yet been investigated. We have previously shown that an AR/EGFR autocrine

loop is functioning in the SUM149 human breast cancer cells and is required for their proliferation, motility and invasive capacity (23). SUM149 cells over express EGFR without gene amplification and the EGFR in these cells is constitutively activated (24). EGFR and AR have been found to be co-over expressed in aggressive breast cancers (25). Thus, it is possible that AR is altering trafficking of the receptor, which could ultimately affect EGFR expression and signaling. Therefore, we investigated degradation of the EGFR and its localization after stimulation with AR.

Body:

Task 2:   To determine how amphiregulin binding to EGFR affects EGFR stability by
comparing the recycling and degradation of EGFR in SUM149 cells and
MCF10A cells stimulated with amphiregulin or EGF:

   a.   Measurement of EGFR half-life in amphiregulin stimulated cells using a
   pulse-chase approach (Months 20-25).

   In order to determine if AR was contributing to the high levels of EGFR
expression observed in SUM149 human breast cancer cells, we measured EGFR
expression by western blot in MCF10A immortalized human mammary epithelial cells
cultured in 10ng/ml EGF (1.7nM), MCF10A cells cultured in 20ng/ml AR (1.8nM)
(MCF10A +AR), MCF10A cells that over express AR (MCF10A AR), and SUM149
cells. The concentration of 20ng/ml AR was used based on previous published results that
demonstrated this concentration was the biological equivalent to EGF for MCF10A cell
proliferation (26). We observed that EGFR protein expression was significantly higher in
SUM149 cells, MCF10A AR cells and MCF10A + AR cells compared with MCF10A
cells cultured in their regular EGF-containing media (Figure 1). Thus, AR activation of
EGFR results in an increase in the steady-state levels of EGFR protein compared with
EGF.

Figure 1
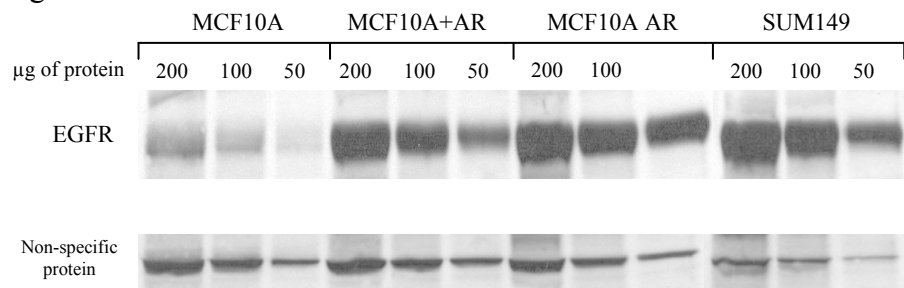
Figure 1: High levels of EGFR protein in SUM149 cells and MCF10A cells with AR activated
EGFR. MCF10A, SUM149, MCF10A AR, and MCF10A+AR cells were lysed and 50, 100, or
200 µg of whole cell lysate protein was separated by SDS-PAGE and then transferred onto a
PVDF membrane. Membranes were probed for EGFR using Zymed monoclonal anti-EGFR
antibody. A non-specific protein band is shown to demonstrate relative loading.

Since we observed increased steady-state levels of EGFR protein in cells that are
dependent on AR, we hypothesized that EGFR degradation might be impaired when AR
is the ligand. In order to determine whether receptor degradation is impaired when
EGFR is activated by AR as compared with EGF, we measured EGFR half-life using
pulse-chase analysis in MCF10A cells cultured in either 10ng/ml EGF or 20ng/ml AR.
MCF10A cells were deprived of methionine and cysteine for 24 hours, pulsed with media
containing ^{35}S labeled methionine/cysteine for 2 hours, and then chased with media

containing excess unlabeled methionine and cysteine for 4, 6, 8, or 10 hours. EGFR was immunoprecipitated and analyzed after gel electrophoresis by autoradiography. Figure 2 demonstrates that in MCF10A cells cultured in the presence of AR, little if any EGFR was degraded by 10 hours. By contrast, most of the labeled EGFR was degraded by 10 hours in the MCF10A cells cultured in EGF containing media. Thus, in the presence of AR, EGFR has a significantly longer half-life which can lead to increases in the steady-state levels of EGFR protein when an AR autocrine loop is functioning in breast cancer.
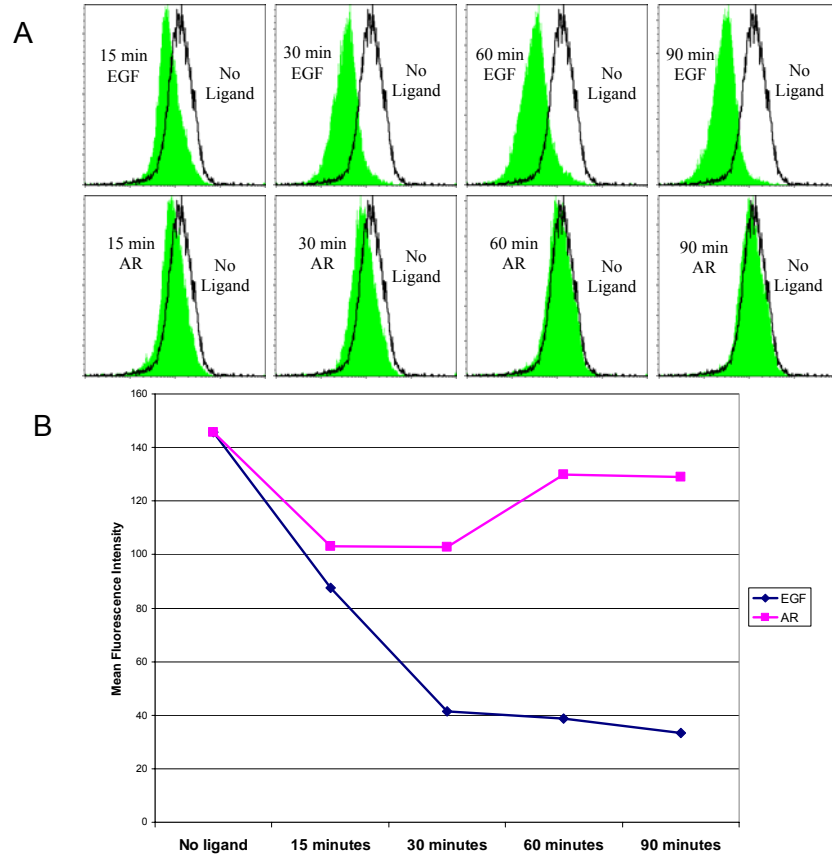
Figure 2
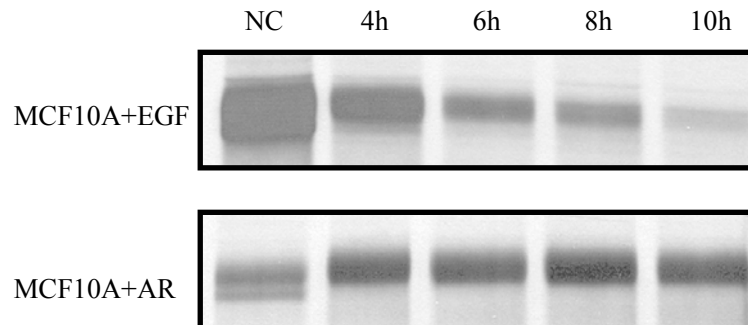
Figure 2: EGFR degradation is decreased in the presence of AR versus EGF during steady-state growth conditions. MCF10A cells were cultured in 10ng/ml EGF (1.7nM) or 20ng/ml AR (1.8nM) for 48 hours and then incubated in methionine/cysteine free media for 1 hour. Cells were pulsed with ^{35}S labeled methionine and cysteine for 2 hours and then chased with media containing 200x more unlabled methionine and cysteine for 4, 6, 8, or 10 hours. NC are cells that were not chased after ^{35}S incorporation.

b. Utilize confocal microscopy to determine whether EGFR is localized in either recycling endosomes or lysosomes after amphiregulin stimulation (Months 25-31).

When stimulated by EGF, EGFR is rapidly endocytosed (16, 27). However, it was not clear whether endogenous EGFR was being internalized in the presence of AR. Therefore, we performed experiments to measure EGF receptor expression on the cell surface following stimulation with AR. MCF10A cells were deprived of EGF for 24 hours and then stimulated with equimolar concentrations of either EGF or AR for 15, 30, 60, or 90 minutes. The cells were then fixed but not permeabilized in order to specifically label EGFR on the cell surface. After fluorescently labeling EGFR on the cells, flow cytometry was performed to quantitate cell surface EGFR on a per cell basis. This assay was used previously in a study that compared surface expression of wild type EGFR and Y1045F mutant EGFR in porcine aortic endothelial cells. In that report, surface wild type EGFR was significantly downregulated after 5 hours of EGF stimulation but surface Y1045F mutant EGFR was unchanged after 5 hours in EGF-containing medium (12). Figure 5 demonstrates that stimulation of MCF10A cells with EGF resulted in rapid internalization of EGFR from the cell surface as demonstrated by the dramatic leftward shift of the peak. In contrast, AR stimulation induced slight

internalization of EGFR with restoration of EGFR cell surface levels by 60 minutes after stimulation. Thus, EGFR in the presence of AR was not effectively internalized compared with EGFR that was activated by EGF.
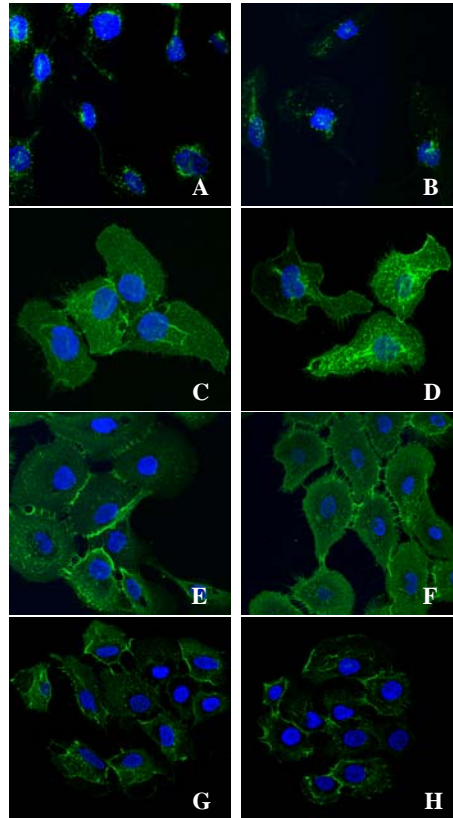
Figure 3:

Figure 3: MCF10A cells were deprived of EGF for 24 hours and then spiked with equimolar concentrations of either EGF or AR (16nM) for 15, 30, 60 or 90 minutes. EGFR on the membrane was fluorescently labeled and analyzed using flow cytometry. A) Flow cytometry graphs where the empty peak represents cell surface EGFR before the addition of ligand and the green peaks represent EGFR after ligand is added. B) A graphical representation of the experiments in 3A indicating mean fluorescence intensity over time.

Previous literature has demonstrated that a majority of the receptors in EGF-stimulated cells can be found in endosomes (11). To determine where in the cell EGFR is localized in the presence of AR, we performed EGFR immunofluorescence studies. MCF10A, MCF10A+AR, MCF10A AR and SUM149 cells were seeded on cover slips and then fixed and permeabilized. The fixed cells were fluorescently labeled and examined on a confocal microscope. We observed that EGFR localization in MCF10A cells cultured in EGF-containing media was predominantly punctate and intracellular (Figure 6). In contrast, EGFR protein in SUM149 cells, MCF10A+AR, and MCF10A AR cells was primarily localized on the membrane (Figure 6). We also observed a concentration of EGFR at points of cell-cell contact in these cells (Figure 7). These results suggest that in MCF10A cells, EGF activation of EGFR induces a predominantly

intracellular localization. However, AR activation of EGFR in MCF10A cells and SUM149 cells appears to alter localization of the receptor so that it is predominantly expressed on the membrane.
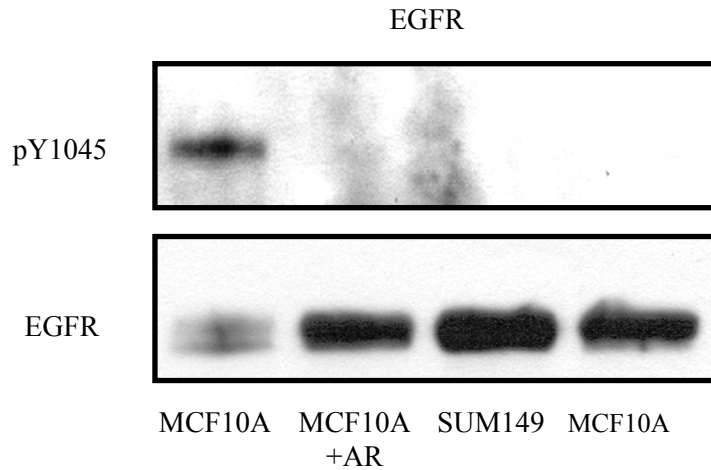
Figure 4

Figure 4: EGFR localization in MCF10A, MCF10A AR, MCF10A + AR, and SUM149 cells. MCF10A (A,B), MCF10A+AR (C,D), MCF10A AR (E,F) and SUM149 (G,H) cells were seeded on coverslips. Cells were then washed, fixed, permeabilized and incubated with anti-EGFR antibody Mab108 for 1 hour followed by incubation with an anti-mouse Alexa 488 conjugated secondary for 1 hour. Coverslips were then mounted on slides and viewed using a 65x water objective on a confocal microscope.

    c. Determine the affect of amphiregulin on EGFR ubiquitination by measuring phosphorylation of tyrosine 1045, the interaction of c-Cbl with EGFR, and ubiquitination of the EGFR after amphiregulin binding (Months 31-36).

       It has been demonstrated that EGFR Y1045 is phosphorylated after EGF activation of EGFR and the ubiquitin ligase c-Cbl binds this phosphorylated Y1045 site on the receptor via its tyrosine kinase binding domain (16). Since we observed decreased degradation of EGFR protein, we next examined phosphorylation of Y1045 in MCF10A, MCF10A+AR, MCF10A AR, and SUM149 cells. Following EGFR immunoprecipitation and western blot analysis, we observed that Y1045 was phosphorylated in MCF10A cells cultured in the presence of EGF. In contrast,

phosphorylation at this site was undetectable in SUM149 breast cancer cells, MCF10A+AR, and MCF10A AR cells (Figure 5).

Figure 5

EGFR

pY1045

EGFR

MCF10A   MCF10A   SUM149   MCF10A
              +AR

Figure 5: Decreased phosphorylation of EGFR Y1045 when AR is the ligand. MCF10A cells cultured in EGF, MCF10A+AR cells, SUM149 cells and MCF10A AR cells were lysed and EGFR was immunoprecipitated using the EGFR.1 antibody (Calbiochem). Membranes were immunoblotted for pY1045 (Cell Signaling) or EGFR (Zymed).

Based on the lack of EGFR Y1045 phosphorylation in AR-stimulated MCF10A cells, we examined ubiquitination of the receptor following EGF or AR stimulation of EGFR. First, MCF10A cells were deprived of EGF for 24 hours to prevent phosphorylation of EGFR and prevent degradation of the receptor. We then stimulated the EGF-deprived MCF10A cells with saturating concentrations of either EGF (16.7nM) or AR (9.09nM) for 5 or 10 minutes. EGFR immunoprecipitation was performed followed by western blot analysis using an anti-ubiquitin antibody that has been used successfully in other studies (22). We observed that after 5 minutes of stimulation with EGF, EGFR exhibited high levels of ubiquitination that began to decrease at 10 minutes. In contrast, AR stimulated low levels of ubiquitination at 5 minutes, which were completely absent by 10 minutes after ligand stimulation (Figure 4). These data are consistent with our pulse-chase data, which showed the enhanced stability of EGFR protein in the presence of AR as compared with EGF.
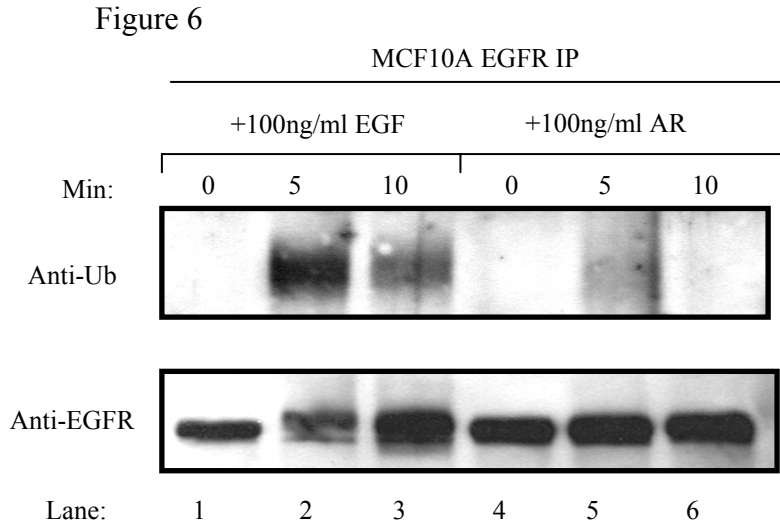
Figure 6

Figure 6: Decreased ubiquitination of EGFR when MCF10A cells are spiked with AR compared with MCF10A cells spiked with EGF. MCF10A cells were deprived of EGF for 24 hours and then stimulated with saturating concentrations of EGF and AR (100ng/ml) for 5 or 10 minutes. Cells were then lysed and EGFR was immunoprecipitated. Membranes were immunoblotted with either an anti-ubiquitin (anti-Ub) antibody (Cell Signaling) or an anti-EGFR antibody (Zymed).

In summary, the above data illustrate that AR activation of EGFR results in increased steady-state levels of the receptor that accumulate at the cell surface. This phenomenon is the result of the lack of phosphorylation of Y1045 on the EGFR, and the resultant failure to ubiquitinate and internalize the receptor in AR-stimulated cells. We propose that accumulation of EGFR at the cell surface alters EGFR signaling in ways that induce motile and invasive properties of breast cancer cells with an AR/EGFR autocrine loop.

Task 3: To determine whether amphiregulin signaling through EGFR contributes to the inflammatory phenotype of Inflammatory Breast Cancer:

    a. Expression profiling of MCF10A cells stimulated by either EGF or amphiregulin using microarray technology followed by QPCR and western analysis to confirm microarray results (Months 4-14).

In an effort to determine which genes might be contributing to the increased cell invasion and motility of MCF10A cells growing with AR stimulated EGFR, we performed an expression array analysis using an Affymetrix human array platform comparing MCF10A cells versus SUM149 cells or MCF10A AR cells. Analysis of the results indicated that 97 genes were increased in their expression in both SUM149 and MCF10A AR cells relative to MCF10A cells (data shown in our previous progress report). Two of these genes, IL-1A and IL-1B were of interest because the IL-1 pathway has been implicated in breast cancer progression (28, 29). IL-1 is a downstream target gene of the transcription factor NF-κB and also a potent inducer of NF-κB activity thus permitting an autoregulatory feedback loop (30, 31). We sought to investigate more

thoroughly this connection between AR and IL-1 to determine whether an IL-1/ NF-κB feedback loop is triggered by AR activation of EGFR.

To investigate the regulation of IL-1 by AR, we measured mRNA and protein expression levels for both IL-1α and IL-1β in SUM149 cells, MCF10A AR cells, and MCF10A cells grown without EGF in the presence of exogenous AR (MCF10A+AR), and compared them to levels in MCF10A cells, grown in the presence of EGF. Figure 7 (A & B) shows that all cell lines in which the EGFR was activated with AR expressed higher mRNA and secreted protein levels of both IL-1α and IL-1β than MCF10A cells grown in EGF. To confirm that IL-1 expression induced by AR depends on EGFR activation, we inhibited EGFR activation in our panel of cell lines using the small molecule EGFR kinase inhibitor Iressa, which effectively inhibits EGFR phosphorylation at a dose of 0.5µM. Figure 7 (A & B) confirms that blocking EGFR activity in SUM149, MCF10A AR, and MCF10A+AR cells resulted in dramatic decreases in mRNA and secreted protein levels of both IL-1α and IL-1β, but had no effect in MCF10A cells cultured in the presence of EGF.
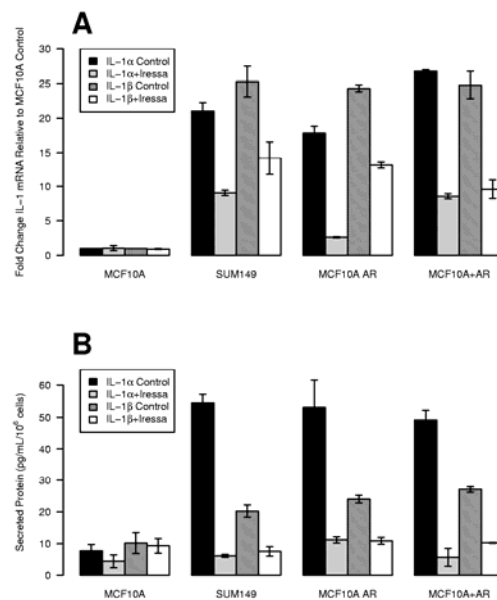
Figure 7


Figure 7: IL-1α and IL-1β mRNA expression (A) and secreted protein levels (B) were measured in MCF10A AR, MCF10A+AR, SUM149, and MCF10A cells by quantitative, real-time PCR and ELISA, respectively. EGFR activation was inhibited by 0.5µM of the kinase inhibitor Iressa. Data are represented as means ± standard error of three independent experiments. Results are shown as fold change relative to MCF10A control.

Having confirmed that AR activated EGFR regulates the expression of IL-1, we next investigated the functional role of the IL-1 pathway on cell proliferation. To inhibit IL-1 signaling, we used recombinant IL-1 receptor antagonist (IL-1ra), which binds the same receptor as IL-1α and β, but does not transduce a signal (32-34). A 10-100 fold molar excess of IL-1ra will effectively block IL-1 signaling and decrease IL-1 secretion (32). To insure the use of an appropriate concentration of IL-1ra, we exposed SUM149

and MCF10A cells to 1, 5, 10, and 20 ng/mL IL-1ra and measured cell proliferation. The data in figure 8A show that inhibition of IL-1 signaling in SUM149 cells dose-dependently decreased cell proliferation in SUM149 cells but had no effect in MCF10A cells grown in the presence of EGF. The growth inhibitory effect of IL-1ra on SUM149 cells was maximal at 10 ng/mL; therefore, we used this concentration for our remaining experiments. Figure 8B shows that cells with over-expression of AR (MCF10A AR), as well as cells grown in the presence of exogenous AR (MCF10A+AR), were also potently growth inhibited by IL-1ra.

The results described above show that AR regulates cell proliferation at least in part through EGFR-mediated up-regulation of IL-1. However, IL-1 has also been shown to stimulate proliferation of cancer cells independent of EGFR activation (35). To investigate the cellular response to increased IL-1 in MCF10A and SUM149 cells without EGFR activation, we examined the ability of SUM149 cells and MCF10A cells to respond to IL-1 in the presence of Iressa. In these experiments, cells were treated with 50 pg/mL IL-1α, 0.5 μM Iressa, or 0.5 μM Iressa + 50 pg/mL IL-1α. The 50 pg/mL dose of recombinant IL-1α was chosen based on the secreted levels of this cytokine measured in SUM149 cells (Figure 7B). Figure 8C shows that both cell types exhibited small increases in proliferation in the presence of IL-1α, suggesting that IL-1 can stimulate proliferation in cells with AR or EGF stimulated EGFR. Interestingly, however, the addition of IL-1α following EGFR inhibition with Iressa resulted in a significant proliferative response of SUM149 cells but not MCF10A cells (Figure 8C). The ability of the SUM149 breast cancer cells to exhibit a mitogenic response to IL-1α in the presence of Iressa could represent a mechanism of resistance to EGFR tyrosine kinase inhibitors, particularly if stromal cells also express and secrete IL-1.
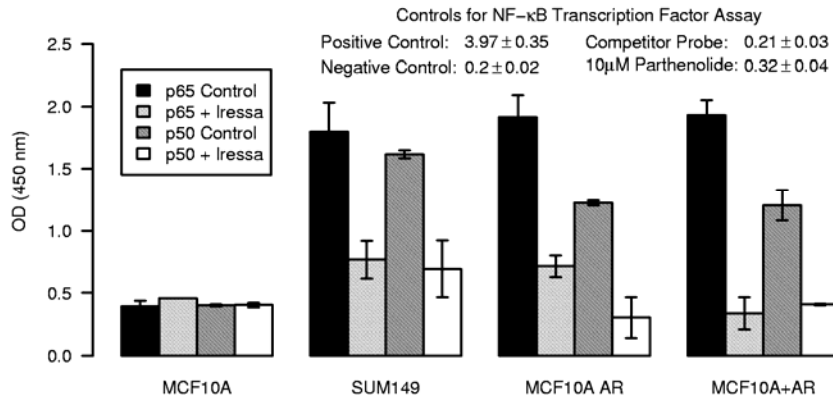
Figure 8
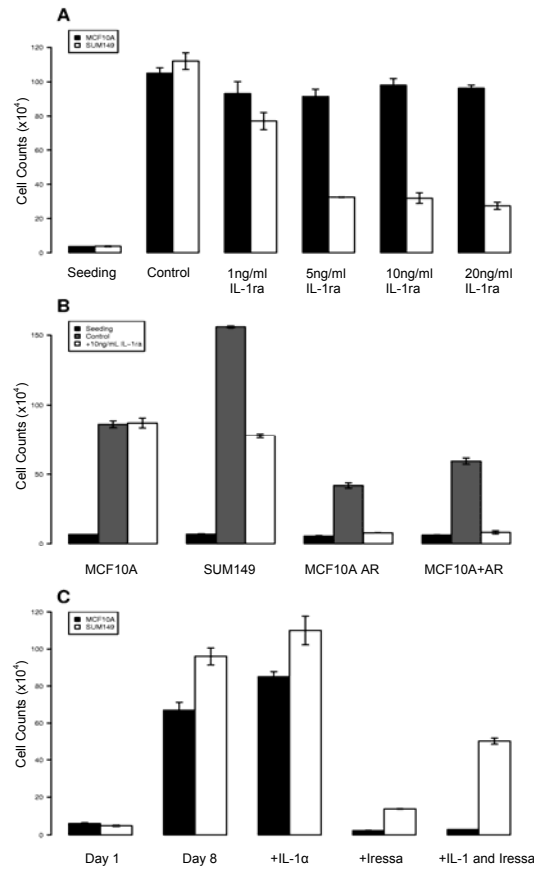
Figure 8: (A) SUM149 and MCF10A cells were incubated with various concentrations of IL-1ra to block IL-1 signaling. Cell counts were determined on days 1 and 8 using a Coulter counter. (B) MCF10A AR, MCF10A+AR, SUM149, and MCF10A cells were treated with 10ng/mL IL-1ra. Cell counts were determined on days 1 and 8 using a Coulter counter. (C) SUM149 and MCF10A cells were treated with 50pg/mL recombinant IL-1α, 0.5 μM Iressa, or 0.5 μM Iressa followed by 50pg/mL recombinant IL-1α. Cell counts were determined on days 1 and 8 using a Coulter Counter. Data are represented as means ± standard error of three independent experiments.

To determine if NF-κB was specifically activated in our AR stimulated cells, we measured NF-κB DNA binding in our panel of cell lines relative to MCF10A control cells cultured in the presence of EGF. To confirm the importance of EGFR signaling in the activation of NF-κB, experiments were performed in the presence or absence of Iressa. Figure 9 shows increased binding of the p50 and p65 NF-κB subunits in the SUM149 breast cancer cells, as well as the MCF10A AR and MCF10A+AR cells, relative to MCF10A cells. Importantly, inhibition of EGFR activity with Iressa completely abrogated the increased p50 and p65 DNA binding observed in cells with AR stimulated EGFR, but had no effect on NF-κB DNA binding in MCF10A cells. These results provide further support for the notion that the relationship between EGFR and IL-1 is distinct in cells with AR stimulated EGFR, as compared to cells with EGF stimulated EGFR.
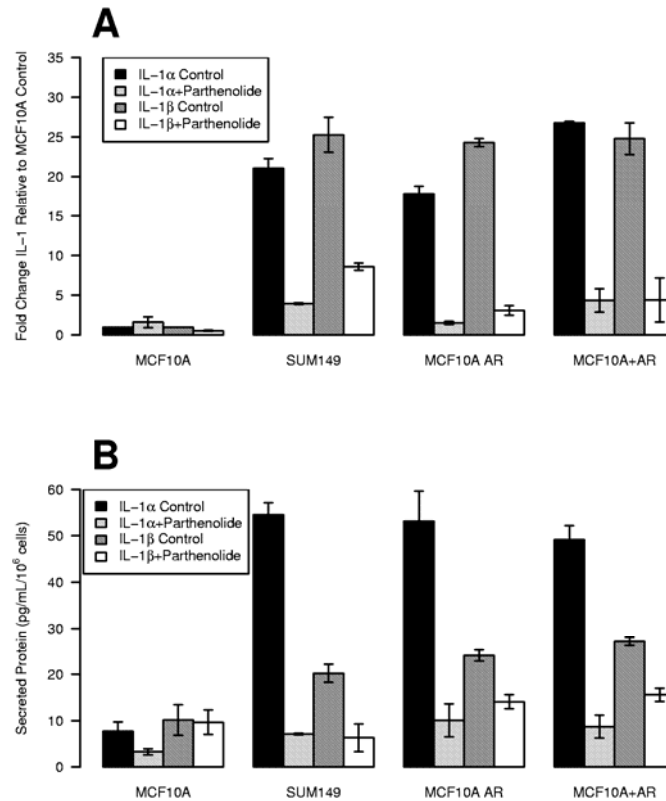
Figure 9

Figure 9: Nuclear extracts from MCF10A AR, MCF10A+AR, SUM149, and MCF10A cells vehicle-treated or treated with 0.5 µM Iressa for 24 hours were collected. A non-radioactive EMSA was used to measure the DNA binding of the p50 and p65 subunits of NF-κB in these samples. Values for a positive control TNF-stimulated HeLa cell extract, a negative control, a competitor probe, and a control following treatment of SUM149 cells with 10µM of the NF-κB inhibitor parthenolide for 8 hours were included to determine the efficacy of the EMSA. Data are represented as means ± standard error of three independent experiments.

To determine if NFκB is the mediator between EGFR and IL-1 synthesis, we treated SUM149, MCF10A AR, MCF10A+AR, and MCF10A cells with 10µM of the NFκB inhibitor parthenolide and collected RNA and conditioned medium. Both IL-1α and IL-1β mRNA expression and secreted protein levels were measured by QPCR and ELISA, respectively. The results shown in figure 10A demonstrated a role of NF-κB in the transcriptional up-regulation of IL-1. Figure 10B shows that NF-κB inhibition decreased the secreted protein levels of IL-1α and IL-1β in all cells except MCF10A, and a similar effect was also observed for intracellular IL-1α protein (data not shown). Together, these results point to a role for NF-κB in regulating the IL-1 pathway downstream of AR stimulated EGFR.

Figure 10:

Figure 10: IL-1α and IL-1β mRNA expression (A) and secreted protein levels (B) were measured in MCF10A AR, MCF10A+AR, SUM149, and MCF10A cells by quantitative, real-time PCR and ELISA, respectively. EGFR activation was inhibited by 10μM of the NF-κB inhibitor parthenolide. Data are represented as means ± standard error of three independent experiments. Results are shown as fold change relative to MCF10A control.

Together, our data are consistent with a model where AR stimulated EGFR activates NF-κB and leads to the transcriptional up-regulation of IL-1. We also have data to support that a feedback loop is initiated where IL-1 feeds back and upregulates NFκB activity (data not shown here, published in manuscript). AR activation of the IL-1 pathway can have clinical implications as far as inhibitor resistance based on the fact that inhibitors of EGFR activity may be relatively ineffective if the IL-1 pathway is also activated and inducing cell proliferation. Additionally, AR induces gene expression changes that are considerably different from EGF and therefore AR could be targeted therapeutically or IL-1 and AR could play potentially important roles as biomarkers for EGFR positive breast cancer.

Key Research Accomplishments:

1. EGFR exhibits decreased downregulation and degradation in the presence of AR versus EGF which contributes to an over expression of EGFR protein on the cell surface.

2. AR induced EGFR leads to increased expression of both IL-1α and IL-1β. In addition, blocking the IL-1 pathway using IL-1 receptor antagonist (IL-1ra) in the presence of AR almost completely blocks proliferation, suggesting that AR induced proliferation is dependent upon IL-1 signaling.

3. AR, but not EGF, stimulated EGFR up-regulates IL-1α and IL-1β through the rapid activation of NF-κB which suggests there is ligand specificity in determining whether NF-κB is activated by EGFR.

Reportable Outcomes:

Manuscripts:

1. Streicher KL*, Willmarth NE*, Garcia J, Boerner JL, Dewey TG, and Ethier SP. Activation of a nuclear factor kappaB/interleukin-1 positive feedback loop by amphiregulin in human breast cancer cells. Mol Cancer Res. 2007 Aug;5(8):847-61.

*these authors contributed equally to the work presented in this paper

2. Willmarth NE and Ethier SP. Altered EGFR localization and degradation in human breast cancer cells with an amphiregulin/EGFR autocrine loop. Cellular Signaling. In Revision.

Abstracts:

1. Streicher KL, Willmarth NE, and Ethier SP. Upregulation of IL-1 by amphiregulin alters EGFR activation by modifying a tyrosine phosphatase. 2007 American Association for Cancer Research (AACR) Annual Meeting in Los Angeles, CA. April 14-18, 2007. (Poster Presentation)

2. Willmarth NE and Ethier SP. EGFR over expression as a result of receptor stabilization in breast cancer cells with an amphiregulin/EGFR autocrine loop. 2007 American Association for Cancer Research (AACR) Annual Meeting in Los Angeles, CA. April 14-18, 2007. (Poster Presentation)

Conclusions:

We have made significant progress in understanding the effect of a functional amphiregulin (AR) autocrine loop in breast cancer progression. We have shown that there is an impairment of EGFR downregulation and degradation when AR is the ligand which contributes to an over expression of EGFR protein on the cell surface. In addition, our studies presented here are the first to indicate that AR specifically activates NF-κB through the EGFR which consequently leads to upregulation of both IL-1α and IL-1β. Since the IL-1 pathway is associated with poorly differentiated and aggressive breast tumors that have an adverse prognosis, the induction of an IL-1/NF-κB positive feedback loop by AR can have important implications on breast cancer progression (28, 29).

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Appendix

Activation of a Nuclear Factor kB/Interleukin-1 Positive Feedback loop by
Amphiregulin in Human Breast Cancer Cells

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Activation of a Nuclear Factor κB/Interleukin-1 Positive Feedback Loop by Amphiregulin in Human Breast Cancer Cells

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Abstract

We have recently shown that an amphiregulin-mediated autocrine loop is responsible for growth factor–independent proliferation, motility, and invasive capacity of some aggressive breast cancer cells, such as the SUM149 breast cancer cell line. In the present study, we investigated the mechanisms by which amphiregulin activation of the epidermal growth factor receptor (EGFR) regulates these altered phenotypes. Bioinformatic analysis of gene expression networks regulated by amphiregulin implicated interleukin-1α (IL-1α) and IL-1β as key mediators of amphiregulin's biological effects. The bioinformatic data were validated in experiments which showed that amphiregulin, but not epidermal growth factor, results in transcriptional up-regulation of IL-1α and IL-1β. Both IL-1α and IL-1β are synthesized and secreted by SUM149 breast cancer cells, as well as MCF10A cells engineered to express amphiregulin or MCF10A cells cultured in the presence of amphiregulin. Furthermore, EGFR, activated by amphiregulin but not epidermal growth factor, results in the prompt activation of the transcription factor nuclear factor–κB (NF-κB), which is required for transcriptional activation of IL-1. Once synthesized and secreted from the cells, IL-1 further activates NF-κB, and inhibition of IL-1 with the IL-1 receptor antagonist results in loss of NF-κB DNA binding activity and inhibition of cell proliferation. However, SUM149 cells can proliferate in the presence of IL-1 when EGFR activity is inhibited. Thus, in aggressive breast cancer cells, such as the SUM149 cells, or in normal human mammary epithelial cells growing in the presence of amphiregulin, EGFR signaling is integrated with NF-κB activation and IL-1 synthesis, which cooperate to regulate the growth and invasive capacity of the cells. (Mol Cancer Res 2007;5(8):847–62)

Introduction

The epidermal growth factor (EGF) receptor (EGFR), or erbB1, is a transmembrane protein (1) possessing intrinsic tyrosine kinase activity. There are several EGF family ligands that can bind and activate EGFR, including epidermal growth factor (EGF; ref. 2), amphiregulin (AR; ref. 3), heparin-binding EGF (HB-EGF; ref. 4), transforming growth factor (TGF)-α (5), epiregulin (6), betacellulin (7), and epigen (8). Ligand binding facilitates dimerization of EGFR, which activates downstream pathways known to be involved in cell growth, proliferation, differentiation, and migration (reviewed in ref. 9). AR was originally purified from the conditioned media of MCF-7 breast cancer cells treated with the tumor promoter phorbol 12-myristate 13-acetate (10). An AR/EGFR autocrine loop has been implicated in cancer progression based on studies using colorectal cancer, pancreatic cancer, and hepatocellular carcinoma cells (11-13). Our laboratory recently discovered that SUM149 inflammatory breast cancer cells have an AR/EGFR autocrine loop that is required for their proliferation, suggesting that an AR/EGFR autocrine loop also plays a role in breast cancer progression (14).

It is apparent from both previous literature and work done in our laboratory that AR activation of EGFR can generate different biological effects on cells and tissues compared with other EGF family ligands. For example, AR but not TGF-α, was able to induce a spindle-like morphology and a relocalization of E-cadherin in Madin-Darby canine kidney cells (15). Also, using targeted knockout mice, Luetteke et al. reported that specific loss of AR, but not EGF or TGF-α, severely stunted ductal outgrowth in the mammary gland (16). Additionally, we have shown that normal MCF10A human mammary epithelial cells exhibit an increased level of cell motility and invasion when stimulated with AR versus EGF (14). Understanding this difference between AR signaling and signaling induced by other EGF family ligands is important to provide more insight into how an AR/EGFR autocrine loop can play a critical role in breast cancer progression.

Our previous studies on the differential effects of EGF versus AR on cell motility and invasion led us to the proinflammatory cytokine interleukin-1 (IL-1). By using microarray expression analysis, we found that AR over-expression in the normal human mammary epithelial cell line MCF10A up-regulates the expression of several genes involved in cell motility and invasion compared with MCF10A cells growing in EGF. Among the genes that were most highly up-regulated by AR were the cytokines IL-1α and IL-1β (14).

The IL-1 cytokines (IL-1α and IL-1β) activate the IL-1RI and are usually secreted only in response to infection or injury. However, IL-1 overexpression, as an autocrine growth factor, has been observed in some cancers (17, 18). IL-1 signaling exerts its effects by regulating the expression of a number of proinflammatory proteins, including growth factors, adhesion molecules, chemokines, and tissue-degrading enzymes (19-22). IL-1, therefore, has been implicated in the regulation of cell proliferation and differentiation, as well as cell motility and invasion. An increased level of IL-1 usually correlates with tumor invasiveness and poor prognosis in cancer patients (reviewed in ref. 23). IL-1 is a downstream target gene of the transcription factor nuclear factor–κB (NF-κB) and also a potent inducer of NF-κB activity, thus permitting an autoregulatory feedback loop (24, 25). The NF-κB proteins [NF-κB$_1$ (p50 and its precursor p105), NF-κB$_2$ (p52 and its precursor p100), RelA (p65), RelB, and c-Rel] are typically sequestered in the cytoplasm and inhibited by IκB, which masks their nuclear localization signal. Upon phosphorylation of IκB by the IκB kinase, IκB is selectively degraded, and consequently NF-κB proteins are released and translocated into the nucleus to activate NF-κB responsive genes (26, 27). IL-1 acts as a potent activator of NF-κB because it induces phosphorylation and, thus, degradation of IκB. An IL-1/NF-κB positive feedback loop has been observed in pancreatic cancer and may also play a role in breast cancer (25).

In this report, we sought to investigate more thoroughly this connection between AR and IL-1 to determine whether an IL-1/NF-κB feedback loop is triggered by AR activation of EGFR. We show that SUM149 inflammatory breast cancer cells, which have an AR/EGFR autocrine loop, are also secreting significant amounts of IL-1α and IL-1β and have increased NF-κB activity, all of which requires EGFR activation. Additionally, we discovered that by overexpressing AR in MCF10A cells or simply growing them in the presence of exogenous AR, an IL-1/NF-κB feedback loop is initiated. Furthermore, inhibition of this feedback loop significantly blocks AR-induced cell proliferation. However, the IL-1/NF-κB feedback loop is not observed when cells are stimulated with EGF instead of AR. Thus, IL-1 signaling is required for AR-induced cell prolifer-ation, suggesting a unique mechanism by which an AR/EGFR autocrine loop can contribute to breast cancer progression.

Results
Activation of EGFR by AR Up-Regulates IL-1

Altered EGFR signaling in breast cancer contributes to increased tumor proliferation and progression. Specifically, we have shown a self-sustaining autocrine loop between EGFR

and its ligand AR in the SUM149 breast cancer cell line that is essential for cell growth and contributes to increased migration and invasion (14). To understand the differential effects of EGFR signaling after activation by EGF or AR, we used two different bioinformatic strategies to identify genes regulated by EGFR activation due to each ligand. First, we used a computational strategy developed by Dewey and coworkers (28-30) that assembles time-series data into phenomenological networks indicative of the specific biolog-ical phenomena regulated by EGFR. The networks obtained using this approach have a scale-free topology, and the hub genes (genes with the highest level of connectivity) in the network are the genes whose expressions are most profoundly affected by blocking EGFR signaling. To map the gene expression networks regulated by AR-stimulated EGFR signaling in SUM149 cells and EGF-stimulated EGFR signaling in MCF10A cells, EGFR activation was inhibited in each cell line with 1 μmol/L CI-1033 for times ranging from 4 to 48 h. mRNA expression was analyzed using Affymetrix U-133a microarrays, and networks were generated using the methods developed by Dewey and coworkers (28-30). Table 1 shows the top 20 hub genes regulated by EGFR signaling and their levels of connectivity in the AR-stimulated SUM149 and EGF-stimulated MCF10A cells. Complete analysis of these networks in the two cell lines will be described in detail in other publications. For the purposes of the present studies, we observed that IL-1α and IL-1β represented major hub genes specific to the EGFR network in SUM149 cells, whereas these genes were not part of the EGFR network in the MCF10A cells with EGF-stimulated EGFR. In a separate, more traditional experimental approach, MCF10A cells, overexpressing AR, were found to express dramatically higher levels of IL-1α and IL-1β than MCF10A cells growing in the presence of EGF (14). The identification of overexpressed IL-1α and IL-1β in two different cell lines with AR-stimulated EGFR using two independent analyses strongly suggests an important role for AR in mediating the activation of IL-1α/β in these cells. Therefore, we designed a series of experiments to further investigate the role of AR-stimulated IL-1 synthesis in the biology of cells responding to AR as an EGFR ligand.

Amphiregulin-Induced EGFR Regulates IL-1

To investigate the regulation of IL-1 by AR, we measured mRNA and protein expression levels for both IL-1α and IL-1β in SUM149 cells, MCF10A cells engineered to overexpress AR (MCF10A AR) cells, and MCF10A cells grown without EGF in the presence of exogenous AR (MCF10A+AR) and compared them to levels in MCF10A cells grown in the presence of EGF. Figure 1(A and B) shows that all cell lines in which EGFR was activated with AR expressed higher mRNA and secreted protein levels of both IL-1α and IL-1β than MCF10A cells, in which EGFR was activated with EGF. To confirm that IL-1 expression induced by AR depends on EGFR activation, we inhibited EGFR activation in our panel of cell lines using the small molecule EGFR kinase inhibitor Iressa, which effectively inhibits EGFR phosphorylation at a dose of 0.5 μmol/L (inset in Fig. 1). Figure 1(A and B) confirms that blocking EGFR activity in

Table 1.   IL-1α and IL-1β Are among the Top Hubs Connected to EGFR in the SUM149 Cell Network

SUM149			MCF10A		
Gene	Weighed Connectivity	Function	Gene	Weighed Connectivity	Function
PHLDA1, pleckstrin homology domain A1	202.9	Apoptosis	CDCA7, cell division cycle associated 7	108.5	Cell proliferation, localization, transport
IL-1α, interluekin 1α	141.7	Cell proliferation, chemotaxis	RRM2, ribonucleotide reductase M2	103.9	DNA metabolism and replication
DIPA, fos-like antigen 1	135.6	Cell proliferation	FLJ11029	97.6	Unknown
IER3, immediate early response 3	87.2	Apoptosis	PSAT1, phosphoserine aminotransferase 1	90.4	Cell metabolism, vitamin biosynthesis
SUPT16H, suppressor of ty 16	78.8	DNA repair	ANLN, anillin	85.8	Cell cycle, mitosis, actin binding
TXNIP, thioredoxin interacting protein	76.5	Oxido-reduction; transcriptional repression	DUSP6, dual specificity phosphatase 6	81.2	Cell cycle, regulates mitogen-activated protein kinase MAPK pathway
EPRS, glutamyl-prolyl-trna synthetase	69.7	RNA metabolism, ligase activity	MAD2L1, mitotic arrest deficient-like 1	81.1	Cell cycle, mitosis
DHX9, deah box polypeptide 9	67.6	RNA helicase activity	C20orf129	73.9	Cell biosynthesis, metabolism
HNRPC, nuclear ribonucleoprotein c	60.8	RNA processing and metabolism	CDC20, cell division cycle 20 homologue	72.3	Cell cycle, mitosis, ubiquitin cycle
MRPS18B, ribosomal protein s18b	60.5	Cell metabolism and biosynthesis	CCNB1, cyclin B1	60.9	Cell cycle, mitosis
MYC, v-myc viral oncogene homologue	60.2	Cell cycle regulation	CDC2, cell division cycle 2	60.5	Cell cycle, mitosis
NUSAP1, nucleolar and spindle associated protein 1	57.4	Mitosis,cytokinesis, spindle localization, cell cycle	KIAA0186, DNA replication complex	59.9	DNA replication and metabolism
DUSP6, dual specificity phosphatase 6	54.3	Cell cycle, MAPK pathway	SCD, stearoyl-coA desaturase	58.9	Biosynthesis, fatty acid metabolism
TOP2A, topoisomerase 2A	53.5	DNA repair and metabolism	CYP1B1, cytochrome p450 1B1	58.1	Cell localization, organ development
JAG1, jagged 1	53.3	Cell migration and proliferation	DIPA, fos-like antigen	57.4	Cell proliferation, transcription
IL-1β, interleukin 1β	51.8	Cell proliferation, response to stress	CKS2, cdc28 protein kinase reg. subunit 2	57.3	Cell cycle, spindle organization
BTF, bcl2-associated transcription factor 1	49.4	Apoptosis, regulates transcription	UHRF1, ubiquitin-like, containing ring finger domains 1	51.7	DNA repair and metabolism, response to DNA damage/stress
CCT2, chaperonin containing tcp1, subunit 2	48.9	Cell cycle, protein folding	NUSAP1, nucleolar and spindle associated protein	50.9	Mitosis, cytokinesis, spindle localization, cell cycle
PLAB, growth differentiation factor 15	44.4	Cell signaling, TGF-β pathway	PHLDA1, pleckstrin homology domain A1	50.1	Apoptosis
FLJ10719	44.1	Unknown	ASNS, asparagine synthetase	48.9	Cell metabolism and biosynthesis

NOTE: Computational network analysis provided data on connectivity of EGFR to genes regulated by this receptor and yielded scale-free networks that identified important hub genes within the EGFR signaling pathway. Hub genes are defined as those genes whose weighed connectivity is in the top 1% of all of the genes in the network and whose expression are most profoundly affected by blocking EGFR signaling. The top 20 hubs in both SUM149 and MCF10A cells are listed and the cancer-specific hubs IL-1α and IL-1β were analyzed further.

SUM149, MCF10A AR, and MCF10A+AR cells resulted in dramatic decreases in mRNA and secreted protein levels of both IL-1α and IL-1β but had no effect in MCF10A cells cultured in the presence of EGF.

Although IL-1α and IL-1β bind to the same receptors, IL-1β is solely active as a secreted protein, whereas IL-1α is active both as an intracellular precursor and as a secreted protein (31, 32). Therefore, we measured levels of intracellular IL-1α in our panel of cell lines under control and Iressa-treated conditions. We found similar patterns as those observed for secreted protein, which is that IL-1α intracellular protein was considerably up-regulated in cells with AR-stimulated EGFR relative to MCF10A cells and this up-regulation was abrogated after EGFR inhibition (data not shown). Together, these data confirm the results obtained from the network analysis and show a distinct role for AR-activated EGFR in regulating the expression of IL-1.

IL-1 Signaling Is Required for Proliferation of Cells with AR-Activated EGFR

Having confirmed that AR-activated EGFR regulates the expression of IL-1, we next investigated the functional role of the IL-1 pathway on cell proliferation. To inhibit IL-1 signaling, we used recombinant IL-1 receptor antagonist (IL-1ra), which binds the same receptor as IL-1α and IL-1β but does not transduce a signal (33-35). A 10-fold to 100-fold molar excess of IL-1ra will effectively block IL-1 signaling and decrease IL-1 secretion (33). To insure the use of an appropriate concentration of IL-1ra, we exposed SUM149 and MCF10A cells to 1, 5, 10, and 20 ng/mL IL-1ra and measured cell proliferation. The data in Fig. 2A show that inhibition of IL-1 signaling in SUM149 cells dose-dependently decreased cell proliferation in SUM149 cells but had no effect in MCF10A cells grown in the presence of EGF (Fig. 2A). The growth
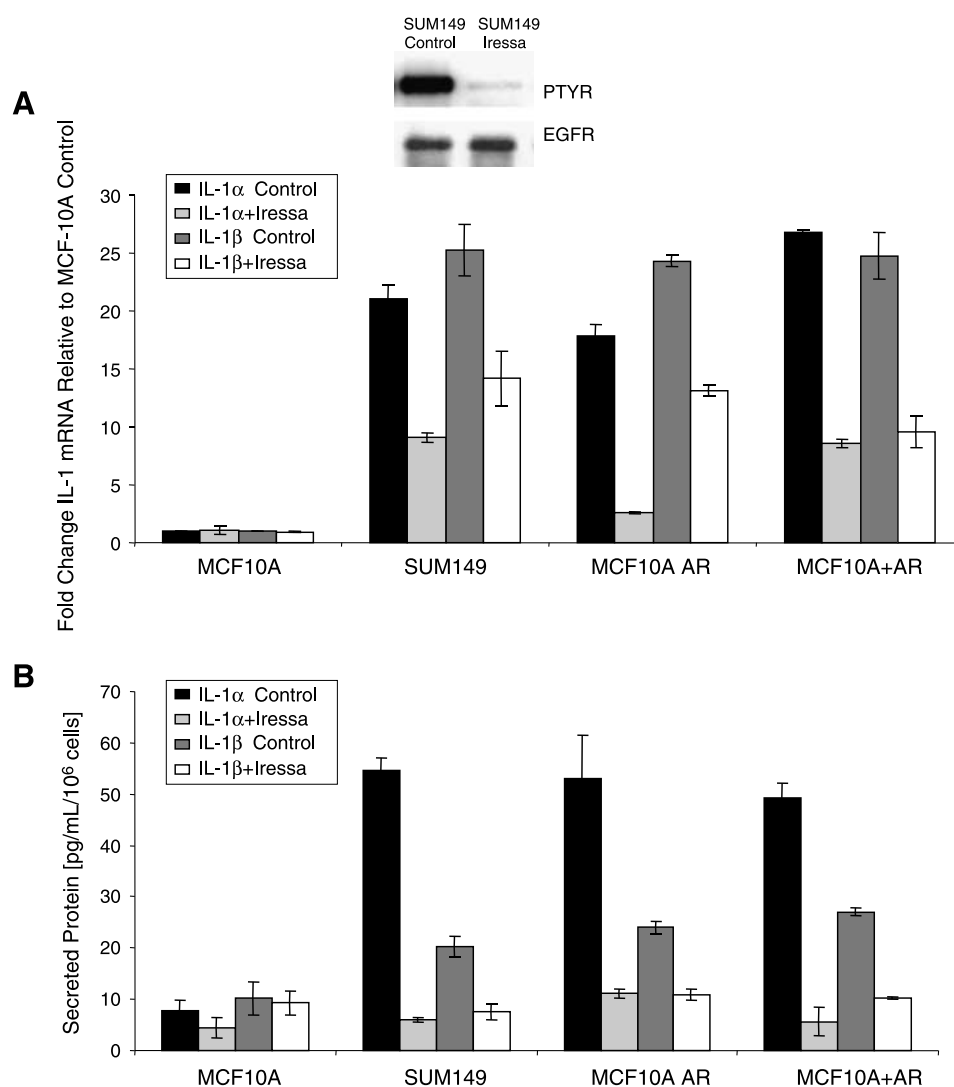
FIGURE 1. EGFR regulates IL-1 mRNA and secretion in cells with AR-stimulated EGFR. IL-1α and IL-1β mRNA expression (**A**) and secreted protein levels (**B**) were measured in MCF10A AR, MCF10A+AR, SUM149, and MCF10A cells by quantitative, real-time PCR and ELISA, respectively. EGFR activation was inhibited by 0.5 μmol/L of the kinase inhibitor Iressa. Columns, means of three independent experiments; bars, SD. Fold change relative to MCF10A control. (An inset confirming the inhibition of EGFR activation by treatment with 0.5 μmol/L Iressa shows an EGFR immunoprecipitation followed by phosphotyrosine and EGFR Western blots in control and Iressa-treated SUM149 cells).

inhibitory effect of IL-1ra on SUM149 cells was maximal at 10 ng/mL; therefore, we used this concentration for our remaining experiments (Fig. 2A). Figure 2B shows that cells with overexpression of AR (MCF10A AR), as well as cells grown in the presence of exogenous AR (MCF10A+AR), were also potently growth-inhibited by IL-1ra (Fig. 2B). The observation that MCF10A cells growing with EGF do not depend on IL-1 for growth shows that AR alters downstream EGFR signaling, leading to increases in IL-1α and IL-1β expression that influence cell proliferation.

The results described above show that AR regulates cell proliferation, at least in part through EGFR-mediated up-regulation of IL-1. However, IL-1 has also been shown to stimulate proliferation of cancer cells independent of EGFR activation (36). To investigate the cellular response to increased IL-1 in our panel of cell lines without EGFR activation, we examined the ability of SUM149, MCF10A AR, MCF10A+AR, and MCF10A cells to respond to IL-1 in the presence of Iressa. In these experiments, cells were treated with 50 pg/mL IL-1α, 0.5 μmol/L Iressa, or 0.5 μmol/L

Iressa + 50 pg/mL IL-1α. The 50 pg/mL as dose of recombinant IL-1α was chosen based on the secreted levels of this cytokine measured in SUM149 cells (Fig. 1B). Figure 2C shows that all cell types exhibited minimal increases in proliferation in the presence of IL-1α, suggesting that IL-1 is not a potent stimulator of proliferation in these cells under normal growth conditions (Fig. 2C). Interestingly, however, the addition of IL-1α after EGFR inhibition with Iressa resulted in a significant proliferative response of SUM149, MCF10A AR, and MCF10A+AR cells, but not MCF10A cells (Fig. 2C). The ability of cells with AR-stimulated EGFR to exhibit a mitogenic response to IL-1α in the presence of Iressa could represent a mechanism of resistance to EGFR tyrosine kinase inhibitors, particularly if stromal cells also express and secrete IL-1.

### NF-κB Binding Activity Is Increased after AR-Induced Activation of EGFR

The results described above validated the link between EGFR, AR, and IL-1 suggested by the network and expression

array analyses and suggested that IL-1 is an important regulator of breast cancer cell growth. Therefore, we did a series of experiments to determine if NF-κB is active in AR-stimulated cells and to examine the relationship between IL-1 expression and NF-κB activation. Accordingly, we measured NF-κB DNA

binding in our panel of cell lines relative to MCF10A control cells cultured in the presence of EGF. To confirm the importance of EGFR signaling in the activation of NF-κB, experiments were done in the presence or absence of Iressa. Figure 3A shows increased binding of the p50 and p65 NF-κB
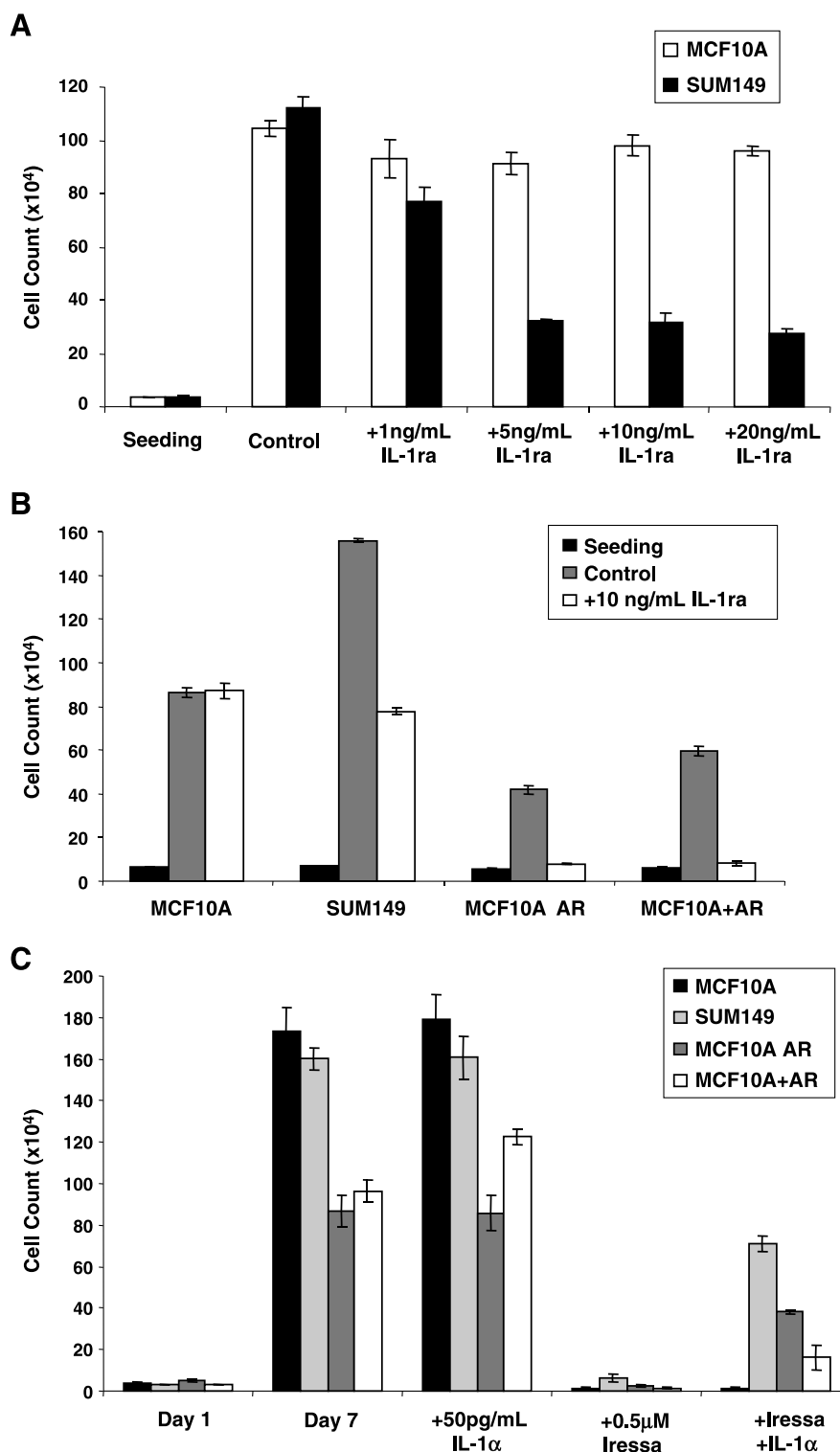
FIGURE 2. IL-1 regulates growth of cells with AR-stimulated EGFR and alters response to exogenous IL-1α. **A.** SUM149 and MCF10A cells were incubated with various concentrations of IL-1ra to block IL-1 signaling. Cell counts were determined on days 1 and 8 using a Coulter counter. **B.** MCF10A AR, MCF10A+AR, SUM149, and MCF10A cells were treated with 10 ng/mL IL-1ra. Cell counts were determined on days 1 and 8 using a Coulter counter. **C.** SUM149, MCF10A AR, MCF10A+AR, and MCF10A cells were treated with 50 pg/mL recombinant IL-1α, 0.5 μmol/L Iressa, or 0.5 μmol/L Iressa followed by 50 pg/mL recombinant IL-1α. Cell counts were determined on days 1 and 8 using a Coulter Counter. Columns, means of three independent experiments; bars, SD.
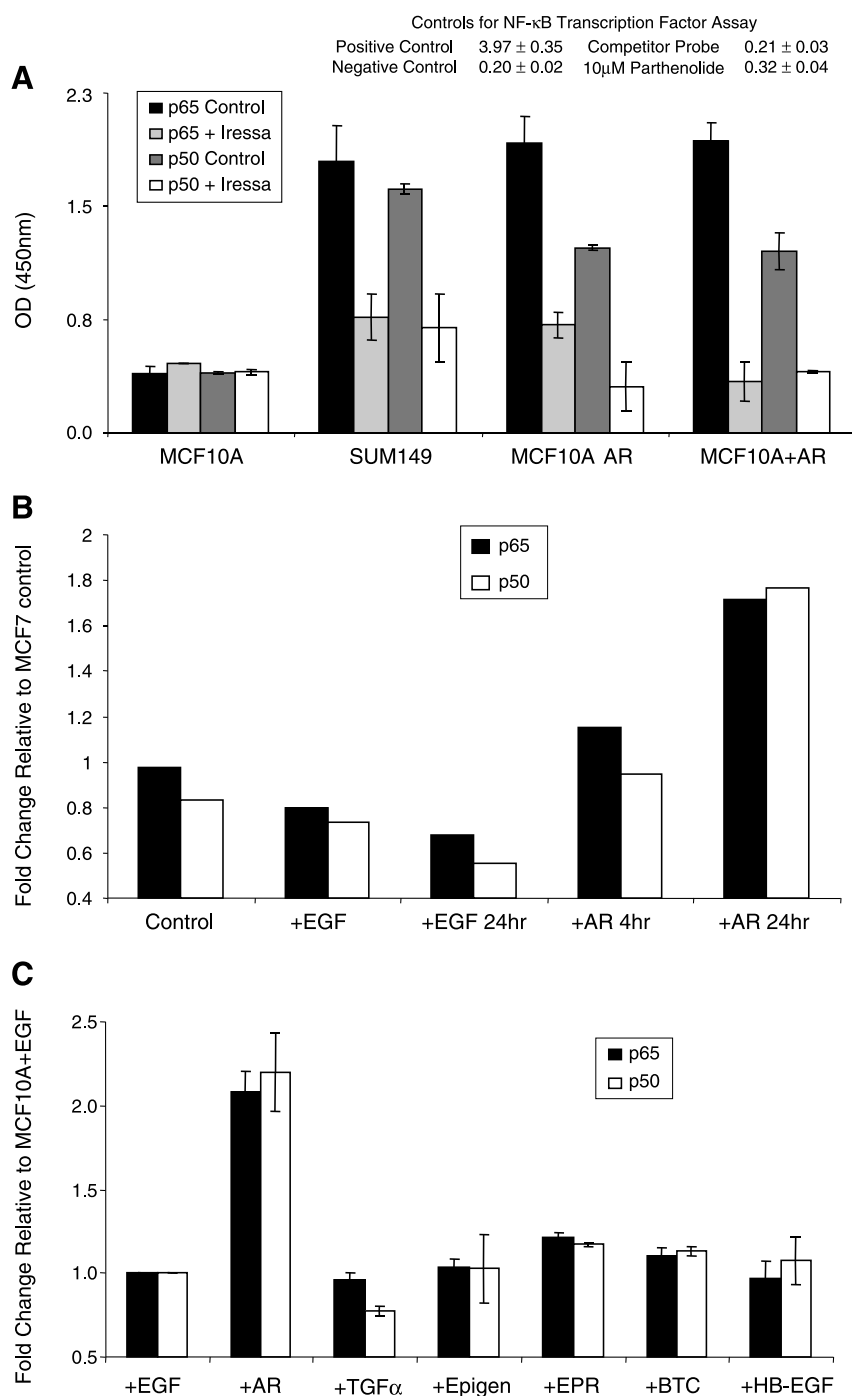
FIGURE 3. AR-stimulated EGFR increases DNA binding of the p50 and p65 NF-κB subunits. Nuclear extracts were collected from the following cells. **A.** MCF10A AR, MCF10A+AR, SUM149, and MCF10A cells vehicle-treated or treated with 0.5 μmol/L Iressa for 24 h. **B.** MCF7 cells engineered to inducibly overexpress EGFR after 24-h stimulation with 2 μg/mL doxacyclin plus 4 nmol/L EGF or 4 nmol/L AR for the indicated time points. **C.** MCF10A cells incubated for 1 h with bioequivalent doses of each of the EGFR ligands: betacellulin (*BTC*; 2 nmol/L), TGF-α (4 nmol/L), HB-EGF (2 nmol/L), epiregulin (*EPR*, 2 nmol/L), epigen (50 nmol/L), AR (4 nmol/L), or EGF (2 nmol/L). A nonradioactive EMSA was used to measure the DNA binding of the p50 and p65 subunits of NF-κB in these samples. Columns, means of three independent experiments; bars, SD. A positive control tumor necrosis factor–stimulated HeLa cell extract, a negative control, a competitor probe, and a control after treatment of SUM149 cells with 10 μmol/L of the NF-κB inhibitor parthenolide for 8 h were included in each run to determine the efficacy of the EMSA. Data for these controls were pooled across all EMSA experiments and presented as the means ± SD.

subunits in the SUM149 breast cancer cells, as well as the MCF10A AR and MCF10A+AR cells, relative to MCF10A cells. Importantly, inhibition of EGFR activity with Iressa completely abrogated the increased p50 and p65 DNA binding observed in cells with AR-stimulated EGFR but had no effect on NF-κB DNA binding in MCF10A cells (Fig. 3A). To determine if the effects of AR on NF-κB DNA binding extend beyond SUM149 and MCF10A AR cells, we also used an MCF7 model with inducible EGFR expression. MCF7 cells were exposed to 2 μg/mL doxacyclin for 24 h to induce EGFR

followed by exposure to EGF or AR for the indicated time points. As shown in Fig. 3B, AR was able to induce NF-κB DNA binding similar to that observed in MCF10A+AR, whereas EGF was unable to induce NF-κB activation, as seen in MCF10A cells.

To determine the specificity of the effects of AR on NF-κB DNA binding, we incubated MCF10A cells for 1 h with bioequivalent doses of each of EGFR ligands, betacellulin (2 nmol/L), TGF-α (4 nmol/L), HB-EGF (2 nmol/L), epiregulin (2 nmol/L), epigen (50 nmol/L), AR (4 nmol/L),

EGF (2 nmol/L), as determined by the ligand concentration, yielding similar growth in MCF10A cells with 2 nmol/L EGF. Figure 3C shows that only AR was able to increase DNA binding of the p65 and p50 NF-κB subunits, which confirms that AR is critical for mediating the observed alterations in EGFR downstream signaling. These results provide further support for the notion that the relationship between EGFR and IL-1 is distinct in cells with AR-stimulated EGFR compared with cells with EGF-stimulated EGFR.

AR-Activated EGFR Signaling Generates a Positive Feedback Loop Involving IL-1 and NF-κB

After confirming that NF-κB DNA binding was decreased after EGFR inhibition, we investigated the role of NF-κB in regulating EGFR-mediated effects on IL-1. To inhibit NF-κB DNA binding, we incubated our panel of cell lines with various concentrations of the NF-κB inhibitor parthenolide and collected nuclear extracts. Parthenolide inhibits NF-κB by two mechanisms: by inhibiting IκB kinase complex and preventing the degradation of IκB-α and IκB-β, which is necessary for translocation of NF-κB to the nucleus (37), as

well as the specific alkylation of cysteine residues within NF-κB subunits (38). In the presence of 10 μmol/L parthenolide, NF-κB DNA binding was dramatically reduced in SUM149, MCF10A AR, and MCF10A+AR cells (Fig. 3). Although 5 μmol/L and 20 μmol/L doses of parthenolide were also tested (data not shown), the 10 μmol/L dose was chosen for future experiments because it inhibited NF-κB DNA binding to levels similar to that seen in MCF10A control cells, and no additional effect was seen with higher concentrations.

We treated SUM149, MCF10A AR, MCF10A+AR, and MCF10A cells with 10 μmol/L parthenolide and collected RNA and conditioned medium. Both IL-1α and IL-1β mRNA expression and secreted protein levels were measured by QPCR and ELISA, respectively. The results shown in Fig. 4A showed a role of NF-κB in the transcriptional up-regulation of IL-1. Figure 4B shows that NF-κB inhibition decreased the secreted protein levels of IL-1α and IL-1β in all cells except MCF10A, and a similar effect was also observed for intracellular IL-1α protein (data not shown). Together, these results point to a role for NF-κB in regulating the IL-1 pathway downstream of AR-stimulated EGFR.
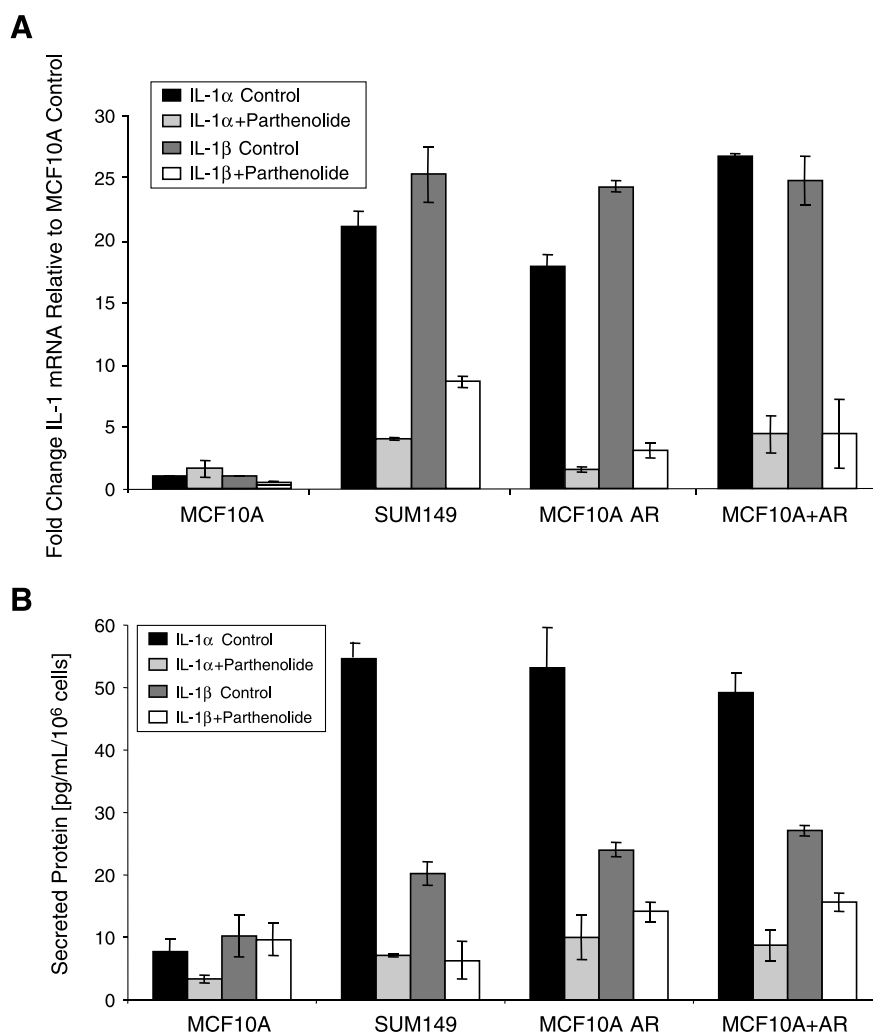
FIGURE 4. AR-stimulated EGFR increases IL-1 mRNA and secretion in an NF-κB–dependent manner. IL-1α and IL-1β mRNA expression (**A**) and secreted protein levels (**B**) were measured in MCF10A AR, MCF10A+AR, SUM149, and MCF10A cells by quantitative, real-time PCR and ELISA, respectively. EGFR activation was inhibited by 10 μmol/L of the NF-κB inhibitor parthenolide. Columns, means of three independent experiments; bars, SD. Fold change relative to MCF10A control.

Because IL-1 is also a well-characterized activator of NF-κB (21, 23, 32, 34, 39) and an IL-1/NF-κB positive feedback loop has been previously shown to affect the development and progression of other cancers (25, 40), we investigated the role of AR in the induction of a similar feedback loop in breast cancer cells. We incubated our panel of cell lines with 10 ng/mL IL-1ra for 8 h, then nuclear extracts were collected and evaluated for NF-κB DNA binding. Figure 5 shows that NF-κB DNA binding was decreased after inhibition of IL-1 signaling in all cells with AR-mediated EGFR activation. Together, our data illustrate that the activation of EGFR signaling by AR generates an active IL-1/NF-κB positive feedback loop, such that disrupting either IL-1 or NF-κB results in loss of activity of the other.
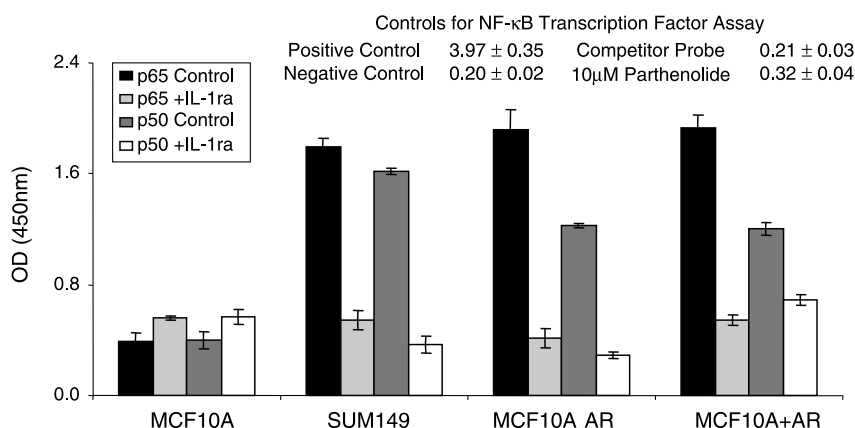
Our data are consistent with a model where AR-stimulated EGFR activates NF-κB and leads to the transcriptional up-regulation of IL-1, as well as a model in which IL-1 up-regulation is induced before NF-κB activation. Therefore, we designed experiments to understand which of these models accurately describes the effects of AR on downstream EGFR signaling. We evaluated changes in NF-κB DNA binding, as well as IL-1α and IL-1β mRNA expression, after a change in ligand from exogenous EGF to AR. Figure 6A shows that NF-κB DNA binding increases within 1 h of the withdrawal of EGF and addition of exogenous AR, and this initial up-regulation is not affected by inhibition of IL-1 signaling. Only at later time points that coincide with the up-regulation of IL-1 does its inhibition have any effect on NF-κB, which is consistent with previous data regarding a feedback loop between these two molecules. Figure 6B shows the results for IL-1α and IL-1β QPCR at multiple time points after the addition of exogenous AR in the presence and absence of the NF-κB inhibitor parthenolide. This figure further supports data in Fig. 6A by showing that IL-1α is not transcriptionally up-regulated by AR until ∼12 h after AR stimulation, and this up-regulation is dependent upon NF-κB activation. Interestingly, IL-1β transcriptional up-regulation is also NF-κB–dependent; however, it does not occur until 24 h after AR exposure, suggesting that IL-1α may also have a role in regulating IL-1β expression (Fig. 6B). Taken together, these data show that NF-κB activation precedes IL-1 transcriptional up-regulation and the development of a feedback loop that is required for

proliferation of cells with AR-stimulated EGFR (Fig. 7). This IL-1/NF-κB interaction induced specifically by AR may represent an important regulatory pathway in a particular subset of breast cancers that could be targeted for therapy.

Discussion

The EGF family ligand AR is overexpressed in ∼50% of all human breast carcinomas, and expression of AR is usually higher in invasive breast carcinomas than ductal carcinoma *in situ* or normal mammary epithelium (41-43). Additionally, expression of antisense AR in a transformed human breast epithelial cell line reduced tumorigenicity of those cells in mice, suggesting that AR plays a very relevant role in breast cancer progression (44). An AR/EGFR autocrine loop has been found to contribute to the progression of several cancers, including non–small cell lung cancer, hepatocellular carcinoma, and colon cancer (11, 13). In agreement, our laboratory has recently shown that the SUM149 inflammatory breast cancer cell line depends on a functional AR/EGFR autocrine loop for proliferation and that AR increases both cellular invasion and motility of human mammary epithelial cells (14).

AR may play a unique role in cancer progression as it has been shown previously to induce differential biological effects through the EGFR compared with other EGF family ligands. For example, the use of knockout mice showed that AR, but not EGF or TGF-α, is required for ductal outgrowth in the developing mouse mammary gland (16). In addition, AR, but not TGF-α, was shown to induce actin rearrangement due to relocalization of E-cadherin in Madin-Darby canine kidney cells (15). Our laboratory has also discovered that AR signaling differs from EGF signaling. MCF10A-immortalized human mammary epithelial cells that overexpress AR or are simply grown in exogenous AR show increased motility and an increased ability to invade compared with MCF10A cells growing in EGF (14). Amphiregulin has a significantly lower affinity for EGFR than EGF and is unable to induce phosphorylation of ErbB-2 in the absence of high levels of EGFR (45, 46). Both ligands are able to induce ErbB-3 phosphorylation to a similar extent, but AR does this in an EGFR-dependent, ErbB-2–independent manner, which is in contrast to the effects observed with EGF (45, 46). The

Controls for NF-κB Transcription Factor Assay

Positive Control	3.97 ± 0.35	Competitor Probe	0.21 ± 0.03
Negative Control	0.20 ± 0.02	10μM Parthenolide	0.32 ± 0.04

FIGURE 5. EGFR activation by AR generates a positive feedback loop between IL-1 and NF-κB. Nuclear extracts from MCF10A AR, MCF10A+AR, SUM149, and MCF10A cells vehicle-treated or treated with 10 ng/mL IL-1ra for 8 h were collected. A nonradioactive EMSA was used to measure the DNA binding of the p50 and p65 subunits of NF-κB in these samples. Columns, means of three independent experiments; bars, SD. A positive control tumor necrosis factor–stimulated HeLa cell extract, a negative control, a competitor probe, and a control after treatment of SUM149 cells with 10 μmol/L of the NF-κB inhibitor parthenolide for 8 h were included in each run to determine the efficacy of the EMSA. Data for these controls were pooled across all experiments and presented as the means ± SD.

A

B

FIGURE 6. AR-stimulated EGFR transcriptionally up-regulates IL-1 due to increased NF-κB activation. **A.** Nuclear extracts from MCF10A, SUM149, and MCF10A cells that were grown in the presence of 20 ng/mL AR for the indicated time points were collected and used in a nonradioactive EMSA to measure DNA binding of the p50 and p65 subunits of NF-κB. Samples were treated also with 10 ng/mL IL-1ra for the indicated time points. Columns, means of three independent experiments; bars, SD. Fold change relative to MCF10A control. **B.** IL-1α and IL-1β mRNA expressions were measured in MCF10A, SUM149, and MCF10A cells that were grown in the presence of 20 ng/mL AR (+AR) for the indicated times by quantitative, real-time PCR. Samples were treated with parthenolide for 4 h at the initial time when MCF10A cells began growing in the presence of exogenous AR, then parthenolide was removed, and for the remainder of the indicated time, cells were grown in +AR medium. Columns, means of three independent experiments; bars, SD. Fold change relative to MCF10A control.
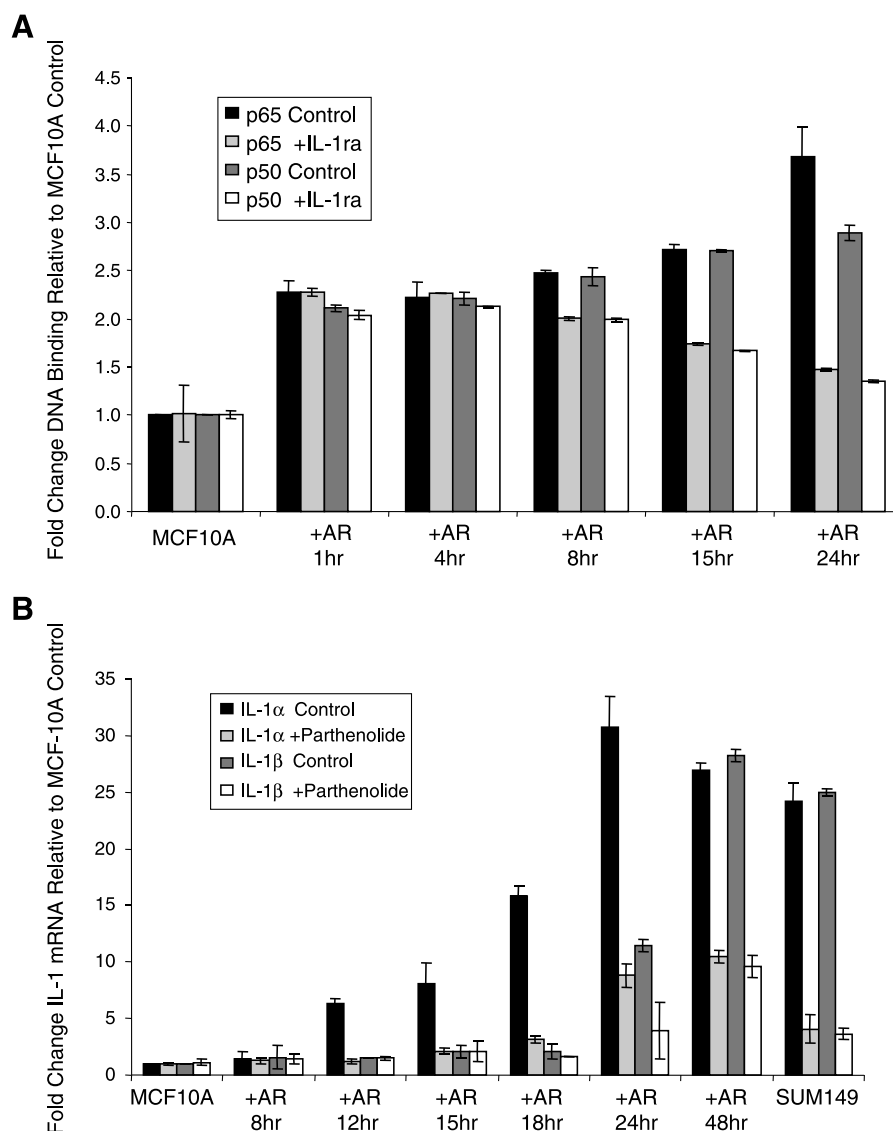
differential effects of AR and EGF on EGFR family members and the possibility that binding of AR to heparin sulfate proteoglycans could promote the formation of dimers between EGFR and ErbB-3 may help to explain the AR-specific alterations in downstream signaling. Although the promotion of EGFR/ErbB3 heterodimers may not be a critical mechanism of AR effects in the SUM149 cells because these cells only have active EGFR, this could play a role in a broader mechanism of AR action in breast cancer cells that express ErbB-3 and should be characterized in future experiments.

The differential effects of EGF and AR signaling on gene expression are not well defined. Therefore, it was important to find genes that may be specifically regulated by AR activation of EGFR in breast cancer cells. Our network analysis identified IL-1 downstream of the EGFR in SUM149 breast cancer cells, which suggested a possible connection between EGFR signaling and IL-1 in the context of AR. The literature showing a connection between IL-1 and EGFR signaling pathways is not

extensive. A link between IL-1 and AR was found previously; however, that study found that IL-1α up-regulated AR expression in cervical cancer cells (22). Our studies presented here are the first to indicate that AR-induced EGFR leads to increased expression of both IL-1α and IL-1β. In addition, blocking the IL-1 pathway using IL-1ra in the presence of AR almost completely blocks proliferation, suggesting that AR-induced proliferation is dependent upon IL-1 signaling. The fact that this effect is observed in the SUM149 breast cancer cells, which we know have an AR/EGFR autocrine loop, suggests that AR up-regulation of IL-1 could occur in other breast cancers with AR/EGFR autocrine loops.

There is recent evidence that suggests a connection between IL-1 and breast cancer. Overexpression of IL-1 in breast cancer cells is associated with poorly differentiated and aggressive breast tumors that have an adverse prognosis (47, 48). IL-1 overexpression has been identified in invasive breast cancers (23, 49), with the highest expression found in ER/PR− cancers,
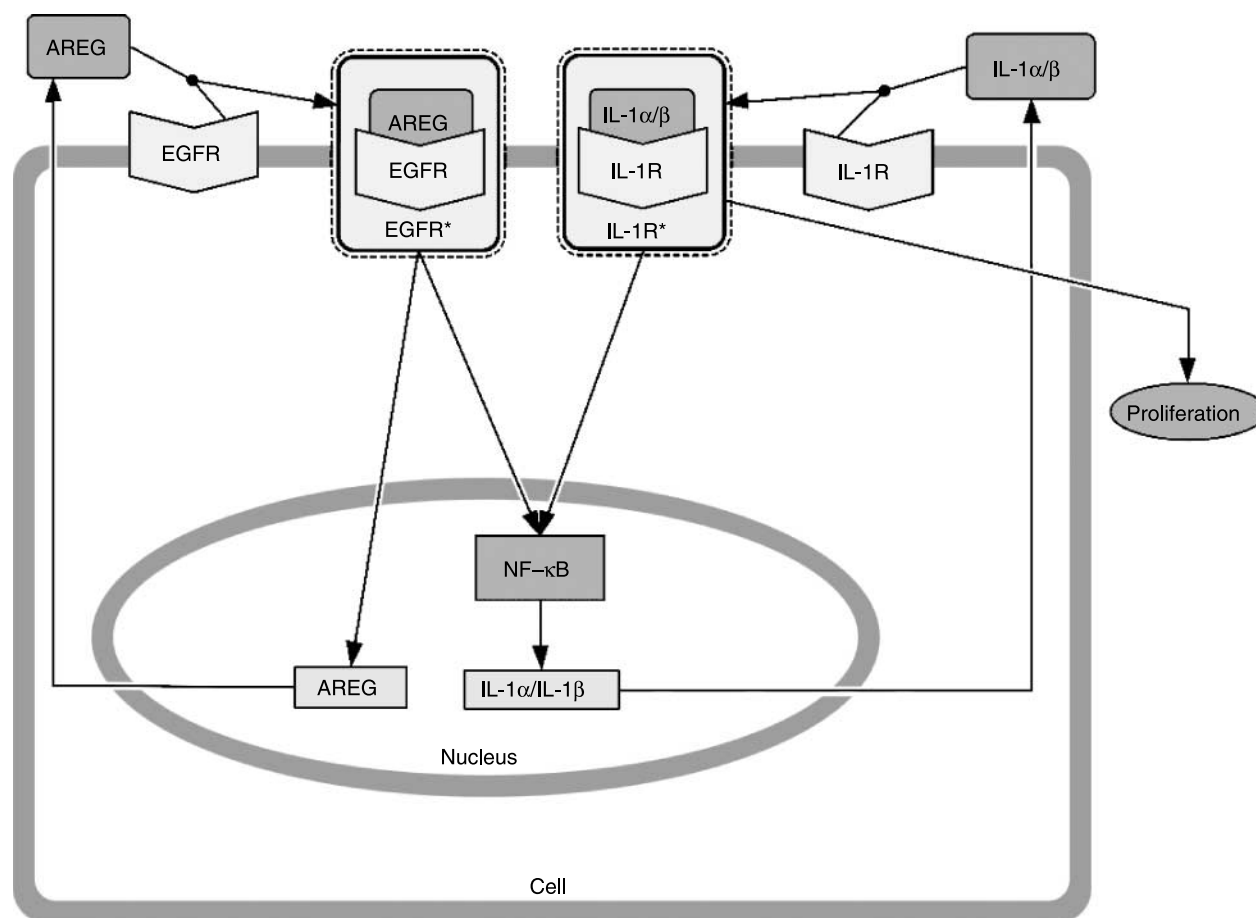
FIGURE 7. Model of the alteration of EGFR downstream signaling due to AR. AR stimulation of EGFR generates a self-sustaining AR/EGFR autocrine loop in which binding of the ligand AR activates EGFR (*EGFR**), which in turn increases the message levels and protein secretion of its ligand AR. Activation of EGFR by AR also up-regulates NF-κB DNA binding, leading to increased IL-1 mRNA expression and secretion, activation of the IL-1 pathway (*IL-1R**), and the generation of an NF-κB/IL-1–positive feedback loop. Both the AR/EGFR autocrine loop and the NF-κB/IL-1–positive feedback loop are critical for the proliferation of cells with AR-stimulated EGFR.

a subset that often exhibits EGFR overexpression (36, 47-50). There are also studies showing that IL-1 signaling increases the expression of matrix metalloproteinase-9, E-selectin, and integrin-1 (19, 20, 51) and enhances tumor cell motility and invasion (50, 52). Thus, it seems that up-regulation of IL-1 is a mechanism by which an AR/EGFR autocrine loop can specifically contribute to the progression of aggressive, invasive breast cancer.

In SUM149 cells with AR-stimulated EGFR, exogenous IL-1 stimulates proliferation even in the absence of EGFR activation; however, this effect is not observed in MCF10A cells with EGF-stimulated EGFR. This specific effect of IL-1 on cancer cells is consistent with a previous work, in which IL-1α stimulated proliferation of cervical cancer cells but not normal cervical cells (22). The effect of IL-1 on cell proliferation has interesting implications for breast cancer progression if IL-1 is present in the tumor microenvironment. Given that IL-1 is produced both by tumor cells and the surrounding stroma (23, 31, 32, 34, 36), the ability of tumor cells to proliferate in response to IL-1 after EGFR inhibition could represent a mechanism of resistance to EGFR inhibitors.

Therefore, inhibiting IL-1 signaling with IL-1ra may be useful in combination with other EGFR inhibitors or other treatment strategies in a subset of breast cancers, in which EGFR activation is driven by AR.

Our data show that AR-stimulated EGFR up-regulates IL-1α and IL-1β through the rapid activation of NF-κB. This is consistent with previous literature showing that EGFR overexpression plays a role in the constitutive activation of NF-κB observed in pancreatic cancer (53), HNSCC (40), smooth muscle cells (54), and several ER−, EGFR+ breast cell lines (54, 55). However, previous research has not fully characterized the role of ligand specificity in regulating that activation of NF-κB by EGFR. The EGFR ligand HB-EGF has been shown to inhibit NF-κB activation in intestinal epithelial cells in a phosphoinositide-3 kinase–dependent manner (56), and TGF-α can induce NF-κB in the vascular wall in response to stress (57). Furthermore, EGF has been implicated in NF-κB activation in fibroblasts due to inter-actions between Grb7 and NF-κB–inducing kinase (58) and in the ER− breast cancer cell lines MDA-MB-231 and MDA-MB-435 cells, although a mechanism describing these

effects was not explored (59). However, we are the first to report the involvement of the NF-κB pathway, specifically downstream of AR-stimulated EGFR in breast cancer. Our findings of a specific regulation of NF-κB by AR in breast cancer cells are in contrast with other studies on HB-EGF and TGF-α in intestinal epithelial cells and vascular endothelium, respectively (56, 57). Additionally, our data also are in contrast with work from Biswas et al., showing that EGF activates NF-κB in ER− breast cancer cell lines (59). Although the reason for this difference is unknown, it is possible that the cells used in this study also produce AR or the addition of EGF leads to increased AR secretion that could be responsible for the observed effects on NF-κB activation. A number of mechanisms have been proposed to explain the activation of NF-κB by EGFR (54, 55, 60). Specifically, Habib et al. have shown that EGFR interacts directly with the key NF-κB signaling proteins, NF-κB–inducing kinase, and RIP (TNFR-interacting protein) to initiate signaling through IκBα and localize NF-κB to the nucleus in MDA-MB-468 breast cancer cells (54). Other studies implicate phosphoinositide-3 kinase in EGFR-mediated NF-κB activation (55, 59, 60). The mechanism of NF-κB activation after AR-stimulated EGFR was not specifically examined in this report but deserves further study.

The identification of NF-κB as a critical component linking EGFR to IL-1 is important because the activation of this transcription factor induces chemotactic genes, growth factors, and matrix metalloproteinases, which are responsible for metastasis, cell proliferation, and progression (61). We showed that the NF-κB subunits p65 and p50 exhibit increased DNA binding in cells with AR-stimulated EGFR, but not those with EGF-stimulated EGFR. The p65 and p50 subunits of NF-κB are most active in epithelial cells (60), and increased DNA binding of these subunits are most often associated with ER− breast cancer; therefore, it is not surprising that the binding activity of these subunits are increased in SUM149 breast cancer cells (62). Currently, treatment of ER/PR−, EGFR+ breast cancers is difficult, as they are typically very aggressive and have not responded well to targeted therapies (59, 60, 63, 64). Interestingly, previous work has shown the regression of EGFR+/ER− breast tumor xenografts after NF-κB inhibition without deleterious side effects (60), as well as the ability of NF-κB and EGFR inhibitors in combination to synergistically block proliferation at concentrations that were ineffective when used individually (60). Although inhibition of NF-κB, specifically in breast cancers with AR-stimulated EGFR, has yet to be fully explored, NF-κB may be a useful biomarker in EGFR+ breast cancer for identifying when combination therapy may be most appropriate.

The induction of an IL-1/NF-κB–positive feedback loop potentiates cancer progression in multiple models (49, 55, 60, 65). Previous research in pancreatic cancer and HNSCC suggests that EGFR overexpression is activating an NF-κB/IL-1 autocrine loop in these cancer cells that is required for cell growth (25, 40). In keratinocytes, the regulation of IL-1 by NF-κB also requires EGFR activation, which is consistent with data presented in this manuscript. AR has been shown to be an autocrine factor in keratinocytes, so it is possible that AR-stimulated EGFR is driving this IL-1/NF-κB feedback loop in the same way as we show in SUM149 breast cancer cells (66). Sustained increases in IL-1 and NF-κB have the ability to alter matrix metalloproteinase activity and the production of other proangiogenic and invasion-inducing factors like IL-6 and IL-8 (40, 52, 67). Accordingly, an active autocrine loop involving IL-1, EGFR, and NF-κB would have the power to induce the rapid growth, invasion, and angiogenesis seen in aggressive breast cancer. Our data show clearly that the prompt activation of NF-κB, induced by simply changing the EGFR ligand from EGF to AR, is required for the transcriptional up-regulation of IL-1 and sufficient to induce generation of this feedback loop involving IL-1 and NF-κB. None of the aforementioned studies examined the specific effects of AR on IL-1 and NF-κB, although the differential EGFR signaling resulting from AR activation could be contributing to the low efficacy of EGFR inhibitors in the clinic.

SUM149 cells were developed from a patient with locally advanced inflammatory breast cancer. Our studies have pointed to a role for AR-activated EGFR in the aggressive growth, motile and invasive phenotype of these cells, which is consistent with the aggressive nature of the patient's disease. Indeed, other studies in breast cancer, pancreas cancer, and other tumor types suggest a role for AR in cancer with aggressive clinical features (11-13, 68). At variance with these observations are recent results reported by Kenny and Bissell, who showed that AR mRNA expression in breast cancer, as detected using gene arrays, did not strongly correlate with EGFR expression, but rather correlated with estrogen receptor expression and a good prognosis (69). At the present time, it is not possible to reconcile these disparate observations, as the two data sets were obtained under completely different conditions. The different results do suggest, however, that simple correlative approaches using AR expression at the message level will not be sufficient to understand the biology of AR in human breast cancer. The results of our experiments reported here and elsewhere indicate that, when HBC cells use AR as an autocrine ligand, EGFR accumulates on the cell surface and EGFR signaling results in expression and activation of IL-1 and NF-κB. Improving our understanding of AR's role in breast cancer will require measurement of all of these biological features associated with AR/EGFR signaling to determine their role in the progression of specific subsets of breast cancer.

Breast cancer is the most commonly diagnosed cancer in women, and EGFR overexpression correlates with a poor prognosis in breast cancer (68). Although there are EGFR inhibitors that are currently being used in the clinic, it is apparent that more research needs to be done to address EGFR inhibitor resistance. AR activation of the IL-1 pathway can have clinical implications for EGFR inhibitor resistance based on the fact that inhibitors of EGFR activity may be relatively ineffective if the IL-1 pathway is also activated and inducing cell proliferation. Additionally, AR induces gene expression changes that are considerably different from EGF and therefore AR could be targeted therapeutically. Alternatively, IL-1 and AR could play potentially important roles as biomarkers for EGFR-positive breast cancer.

Materials and Methods

Network Analysis

Gene expression networks from SUM149 and MCF10A cells were determined from an analysis of global gene expression time series data. MCF10A and SUM149 cells were cultured to ~75% confluence and exposed to EGFR inhibitor CI-1033. RNA was isolated from cells at 0, 4, 8, 12, 16, 24, and 48 h after addition of drug, and corresponding gene expression levels were determined using Affymetrix U-133a and U-133b microarrays. Microarrays were analyzed using standard procedures. For filtering purposes, only genes showing a fold change in its time series of >1.7 were considered for further analysis. Cubic spline interpolation was used to calculate expression levels at 4-h time intervals. To calculate the network associated with each time series, a linear finite difference model was used as described previously (28-30). This model assumes that the gene expression levels of a given gene at a given time depend upon the expression levels of other genes at a single previous time. A discrete linear response model is used to generate a transition matrix calculated from the experimental data using matrix inversion procedures (28-30). Unitless variables are generated from these matrices, which represent phenomenological variables that show how the expression level of a gene at one point influences the expression level of another at a later time point. Biological systems are generally nonlinear, and it is not presumed that such a simple linear response model will fully capture the underlying causal network of gene expression. However, this model is designed as a data-mining tool to capture the phenomenological influence of one expression level on another. The resulting networks have a scale-free topology and display a hub-and-spoke pattern that follows a power law distribution. Two network variables, clustering coefficient and the characteristic path length, were determined from the networks and were used to determine overall connectivity of the genes, which also determines the genes identified as hubs. We identified hub genes because a strong relationship has been shown between a molecule's hub status and its importance in maintaining appropriate cellular function (70). Appropriate biological assays were used to validate the results of the network analysis.

Cell Lines and Cell Culture Conditions

The MCF10A human mammary epithelial cell line is cultured in SFIHE medium [Ham's F-12 medium supplemented with 0.1% bovine serum albumin, fungizone (0.5 μg/mL), gentamicin (5 μg/mL), ethanolamine (5 mmol/L), HEPES (10 mmol/L), transferrin (5 μg/mL), 3,3′,5-triiodo-L-thyronine (10 μmol/L), selenium (50 μmol/L), hydrocortisone (1 μg/mL), insulin (5 μg/mL), and 10 ng/mL EGF]. SUM149 cells were maintained in 5% IH (Ham's F-12 with 5% fetal bovine serum, supplemented with insulin, hydrocortisone, fungizone, and gentamicin at the same concentrations as for MCF10A cells). Complete culture conditions for both cell lines were as described previously (71). MCF10A AR cells were grown in the same culture media as MCF10A cells but without EGF (SFIH). MCF10A+AR cells were grown in the same media as MCF10A AR cells with addition of 20 ng/mL recombinant AR (R&D Systems; SFIHA). Other EGFR ligands were used at the following doses: betacellulin, 2 nmol/L; TGF-α, 4 nmol/L;

HB-EGF, 2 nmol/L; EREG, 2 nmol/L; epigen, 50 nmol/L; AR, 4 nmol/L; EGF, 2 nmol/L. All cells were cultured at 37°C in a humidified incubator containing 10% CO_2 and were maintained free of *Mycoplasma*.

MCF7 breast cancer cells stably overexpressing EGFR under control of the Tet-ON vector were a generous gift from by Dr. Julie Boerner and generated as previously described (72). To generate these cells, a pTRE2 vector encoding *wt*-EGFR was transfected together with a pBabe-puro vector for antibiotic selection of stably expressing clones. Clonal isolates were recloned until cell lines that inducibly expressed EGFR constructs in 80% to 90% of the cells (as determined by immunofluorescence) were obtained. This cell line is maintained in DMEM with 10% fetal bovine serum, 1 mmol/L sodium pyruvate, 100 μg/mL G418, and 10 μg/mL puromycin. EGFR expression in induced by the addition of 2 μg/mL doxacyclin for 24 h.

Nuclear Extracts and Whole-Cell Lysates

Cells were rinsed twice with ice-cold HBSS (Life Technologies) and then lysed on ice with a buffer consisting of Tris-HCl (50 mmol/L, pH 8.5), NaCl (150 mmol/L), 1% NP40 (ICN Biomedical, Inc.), EDTA (5 mmol/L) supplemented with sodium orthovanadate (5 mmol/L), phenylmethsulfonyl fluoride (50 μg/mL), aprotinin (20 μg/mL), and leupeptin (10 μg/mL). Lysates were spun at 14,000×g at 4°C for 10 min and then analyzed for protein using the Bradford method (Bio-Rad Laboratories).

Nuclear extracts were isolated using the NE-PER extraction kit according to manufacturer's instructions (Pierce). Briefly, cell pellets were lysed with hypotonic lysis buffer, including DTT and protease inhibitors. Cells were incubated on ice for 15 min before adding 10% IGEPAL CA-630 solution to the swollen cells. Cells were centrifuged for 30 s at 11,000×g, and the supernatant was transferred to a fresh tube (cytoplasmic fraction). The remaining pellet was resuspended in extraction buffer, including DTT and protease inhibitors. Tubes were vortexed for 15 s every 10 min for 40 min then centrifuged for 5 min at 21,000×g. Supernatant was transferred to new tube and stored at -70°C.

IL-1α/β Enzyme–Linked Immunosorbent Assay

MCF10A and SUM149 cells were untreated or treated for 24 h with 0.5 μmol/L Gefitinib/Iressa (AstraZeneca Pharmaceuticals), 10 ng/mL IL-1ra (Cell Sciences), or 10 μmol/L NF-κB inhibitor parthenolide (Alexis Biochemicals). Conditioned medium and whole-cell lysates from each of these treatment groups were collected to evaluate the secreted and cellular protein levels of IL-1α, respectively. Conditioned medium from these treatment groups was also analyzed for secreted levels of IL-1β.

IL-1α/β ELISAs were done using commercially available DuoSet ELISA development kits (R&D Systems) following the manufacturer's instructions. Briefly, high-binding ELISA 96-well plates were coated with IL-1α or IL-1β capture antibody overnight at room temperature. Absorbance at 450 nmol/L minus the absorbance at 550 nmol/L was measured on a VERSAmax microplate reader (Molecular Devices Corp.). A standard curve of known concentrations of recombinant IL-1α

or IL-1β was generated for each ELISA by plotting the log of the IL-1α or IL-1β concentration versus the log of the absorbance reading and used to quantify the concentration of IL-1α or IL-1β in each sample. Cells were lysed and nuclei were counted with a Coulter Counter (Beckman Coulter) for normalization.

Assessment of Monolayer Growth

Cells were seeded into six-well plates at 3.5×10^4 per well in SFIHE (plus 2% fetal bovine serum to allow attachment) or 5% IH or SFIH media. The next day, plating medium was removed, and cells were treated with SFIH (MCF10A AR), SFIHE (MCF10A), SFIHA (MCF10A+AR), or 5% IH (SUM149) ± 10 ng/mL IL-1ra, 0.5 μmol/L Iressa, or recombinant IL-1α for 7 days, with fresh treatment added everyday. The number of cells was determined by counting isolated nuclei with a Coulter counter 7 days after treatment. A plating efficiency was done 24 h after plating to determine the number of attached cells per well. All experiments were done in triplicate and repeated at least twice.

NF-κB Transcription Factor Assay

To evaluate the DNA binding of the p50 and p65 subunits of NF-κB, we used a transcription factor assay kit combining an electrophoretic mobility shift assay with an ELISA assay according to manufacturer's instructions (Chemicon International). Briefly, a double-stranded biotinylated oligonucleotide containing the flanked DNA binding consensus sequence for NF-κB (5′-GGGACTTTCC-3′) is mixed with nuclear extract in the provided transcription factor assay buffer. During this incubation, the active form of NF-κB in the nuclear extract binds to its consensus sequence. The extract/probe/buffer mixture is then directly transferred to a strepavidin-coated 96-well plate. The active NF-κB protein immobilized on the biotinylated double-stranded oligonucleotide capture probe binds to the strepavidin plate well, and any inactive, unbound material is washed away. The bound NF-κB transcription factor subunits, p50 and p65, are detected with specific primary antibodies, a rabbit anti–NF-κB p50 and a rabbit anti–NF-κB p65. A highly sensitive horseradish peroxidase–conjugated secondary antibody is used for colorimetric detection that is read in a spectrophotometric plate reader. To insure specific NF-κB binding, a positive control (tumor necrosis factor–stimulated HeLa cell extract), nonspecific double-stranded oligonucleotide, and a specific competitor double-stranded oligonucleotide are included in each assay.

Q-Reverse Transcription–PCR Reactions

RNA was extracted from SUM149, MCF10A, MCF10A+AR, and MCF10A AR cells using the Qiagen RNeasy kit. RNA was converted into cDNA via a reverse transcription reaction using random hexamer primers. IL-1α and IL-1β probes were ordered from Applied Biosystems Assays-by-Design service. A glyceraldehyde-3-phosphate dehydrogenase primer set was used as a control. RNA (2 μg) was used for the reverse transcription–PCR reaction, and the product was diluted at 1:12. Q–reverse transcription–PCR was done in 25-μL reactions in 96-well plates using the Taqman Universal PCR

Master Mix (Applied Biosystems). Reactions were done twice, in replicates of three or four, using the Bio-Rad iQ5 real-time PCR machine (Bio-Rad Laboratories). Cycles to threshold values for IL-1α and IL-1β were normalized to values for glyceraldehyde-3-phosphate dehydrogenase then compared with IL-1α and IL-1β expression in MCF10A cells. Control wells containing PCR master mix and primers without sample cDNA emitted no fluorescence after 40 cycles. Relative expression data was calculated as described by Livak and Schmittgen (73). Briefly, average values were determined for number of cycles in each reaction to achieve a threshold of fluorescence. The average numbers of cycles necessary for the glyceraldehyde-3-phosphate dehydrogenase reaction were subtracted from these values, followed by subtraction of the average cycle numbers in a control cell line, in this case MCF10A. The fold difference was determined by raising 2 to the negative power of the calculated difference.

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